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COORDINATORE Prof. Persio Dello Sbarba

Protective role of antioxidants in bone and bowel pathological alterations related to oxidative stress

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Dottoranda
Dott.ssa Vladana Domazetovic

Tutore
Prof.ssa Maria Teresa Vincenzini

Coordinatore
Prof. Persio Dello Sbarba

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(Domazetovic et al., 2017)

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ABBREVIATIONS

17β-E2	17-β-estradiol
ADAM	A disintegrin and metalloproteinase domain
AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride
ALP	alkaline phosphatase
AOM	azoxymethane
APP	amyloid precursor protein
BB	blueberry
BCA	bicinhoninic acid
BE	blueberry extract
BJ	blueberry juice
BMD	bone mineral density
BSO	buthionine-(S,R)-sulfoximine
CD	Crohn's disease
COX-2	cyclooxygenase-2
CSF-1	colony stimulating factor 1
CYP	cytochrome P450
Dmp1	dentin matrix acidic phosphoprotein 1
DMSO	dimethyl sulfoxide
DNIC	dinitrosyl iron complex
DPI	diphenyleneiodonium chloride
DSS	dextran sulfate sodium
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EGTA	ethylene-bis(oxyethylenenitrilo)tetraacetic acid
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FADD	Fas associated death domain protein
FGF	fibroblast growth factor
GI	gastro-intestinal
GPX	glutathione peroxidase

GSH	reduced glutathione
GSR	glutathione reductase
GSSG	oxidized glutathione
GST	glutathione S-transferase
GT	green tea
GTE	green tea extract
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HE	Hypericum perforatum extract
HMG	high mobility group box 1
HO	heme oxygenase
HP	Hypericum perforatum
IBD	inflammatory bowel disease
ICAM-1	intracellular adhesion molecule 1
ICC	interstitial cells of Cajal
IDO	indoleamine 2,3-dioxygenase
IEC	intestinal endothelial cells
IGF	insulin-like growth factor
IKK	kB inhibitor
IL	interleukin
ISEMF	intestinal sub-epithelial myofibroblast
JNK	c-Jun N-terminal kinase
LA	lipoic acid
LDL	low density lipoprotein
MAPK	mitogen-derived protein kinase
MDA	malondialdehyde
MHC	major histocompatibility class
Mito TEMPO	2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl-triphenylphosphonium chloride monohydrate
MKP-1	mitogen-activated protein kinase phosphatase-1
MMP	metalloproteinase
NAC	N-acetylcysteine
NCAM	neural cell adhesion molecule
NF-κB	nuclear factor kappa-B

NK-1	neurokinin-1
NOS	nitric oxide synthase
NOX	NADPH oxidase
OPG	osteoprotegerin
PAMPs	pathogen-associated molecular patterns
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase type I
PKC	protein kinase C
PMNL	polymorphomononuclear leukocytes
Prx	peroxiredoxins
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone
PVDF	polyvinylidene difluoride
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RE	resveratrol
RIP	serine/threonine receptor interacting with protein kinase 1
RNS	reactive nitrogen species
ROS	reactive oxygen species
RUNX-2	Runt-related transcription factor 2
SERM	selective estrogen receptor modulators
SFRP	selected frizzled related protein 1
SOD	superoxide dismutase
SP	substance P
TACE	tumor necrosis factor-converting enzyme
TAPI-1	tumor necrosis factor alpha protease inhibitor-1
TGFβ	transforming growth factor beta
Th	T helper
TIMP-1	tissue inhibitor of metalloproteinases 1
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNFα	tumor necrosis factor alpha
Tpl2	tumor progression locus-2
TRADD	tumor necrosis factor receptor associated death domain

TRAF2	tumor necrosis factor receptor-associated factor 2
Treg	T regulatory
Trx	thioredoxin
TrxR	thioredoxin reductase
TSP	total soluble polyphenol fraction
UC	Ulcerative colitis
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
α-SMA	α-smooth muscle actin

INTRODUCTION

1. Intracellular ROS production: physiological role and oxidative stress

Oxidizing agents are generated during physiological cell processes, such as oxidative phosphorylation that takes place in the mitochondria and is a major source of ATP in aerobic organisms, catalytic activity of most enzymes, and protein folding in which free sulfhydryl groups are oxidized and stabilized as disulfide bonds. By-products of these processes are free radicals that include reactive oxygen species (ROS), reactive nitrogen species (RNS), carbon-centered and sulfur-centered radicals [Pero et al., 1990]. The process of reduction of molecular oxygen to water generates ROS such as superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). Superoxide is the least reactive of the three ROS species, however it can be converted by superoxide dismutase (SOD) to H_2O_2, which further decomposes to yield $OH^{\cdot}$ in the presence of iron. This latter ROS is extremely reactive specie and capable of interacting closely to its site of formation with a wide range of biological molecules such as fatty acids, nucleic acids, proteins, enzymes, polysaccharides. RNS include nitric oxide radical ($NO\cdot$), relatively unreactive gaseous radical of biological interest, and peroxinitrite ($ONOO^-$), highly reactive non-radical species formed from the nitric oxide and superoxide anion radicals.

It is a newly appreciated concept that ROS and RNS function as signalling molecules that modulate a variety of intracellular processes (Fig. 1). It is only around the '90s that it has been seen that some ROS species are required for cytokine, insulin, growth factor and NF-κB signalling [Finkel, 1998] and that $NO\cdot$ is required for blood vessel relaxation [Ignarro et al., 1987]. ROS can cause reversible post-translational protein modifications changing their activity and the related signalling pathways. This is of great importance for kinases, phosphatases and transcription factors. For example, phosphatases are more likely to be susceptible to ROS regulation, as they possess a reactive cysteine in their catalytic domain that can be reversibly oxidized thus blocking temporarily their dephosphorylating

activity [Rhee et al., 2000]. ROS are involved in programmed cell death process called apoptosis, important for maintenance of cellular homeostasis during development and aging, as well as for removal of the damaged cells. Moreover, other processes important for maintenance of homeostasis such as autophagy and mitophagy (autophagy of mitochondria), are also regulated by ROS levels. In immune cells they are best known for their role in eliminating pathogens through the oxidative burst in phagosomes, and appear to be essential for a wide range of innate immune functions such as antiviral, antibacterial and antiparasitic responses. In other cell types, ROS can mediate inflammatory cytokine signalling. Data regarding the differentiation of human stem cells also indicate the importance of ROS in the metabolic state and cell fate of these cells.

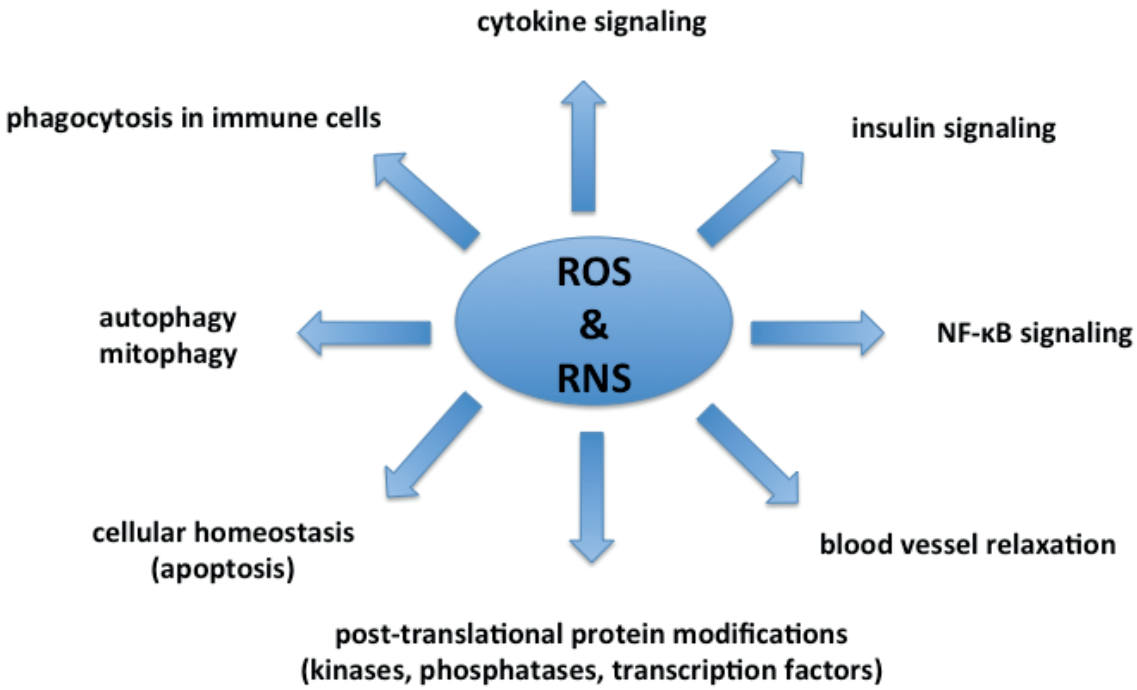


Figure 1. Physiological role of ROS.

In living organisms, reactive species are generated in several cell compartments such as plasma membrane, mitochondria, cytosol, peroxisomes, lysosomes and endoplasmic reticulum. While for ROS the situation is rather simple, for RNS it becomes complicated and still under debate for certain reasons.

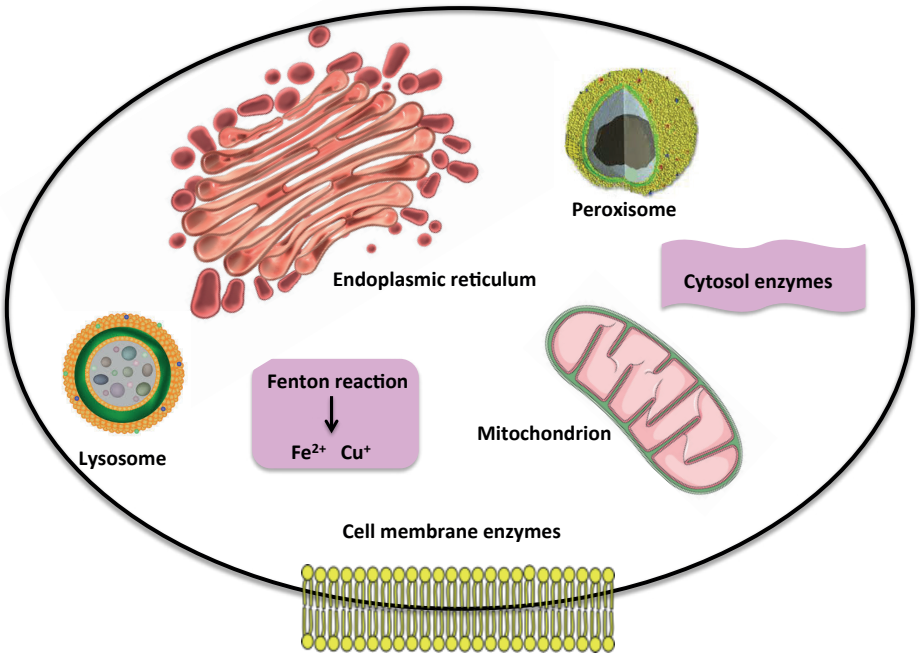


Figure 2. Intracellular sources of ROS production.

The main source of ROS production occurring on plasma membrane is represented by NADPH oxidase (NOX) enzymes. For many years NOX was thought to exist only in phagocytes, given its fundamental role in eliminating bacterial intruders. Afterwards, several enzymes similar to phagocytic NOX have been identified in various tissues and referred as NOX family. Two integral membrane proteins that together form a large heterodimer subunit, along with four cytosolic subunits that have regulatory role, form NOX inactive complex. Upon stimulation, the cytosolic subunits translocate to the membrane and associate with the membrane heterodimer subunit in order to form the active oxidase. This process is tightly regulated by phosphorylation of cytosolic regulatory units [Yamamori et al., 2004; Chen et al., 2014], by protein-protein interactions between NOX and

members of thioredoxin family and by intracellular concentration of various ions [Janiszewski et al., 2005; Pandey et al., 2011]. NOX enzymes possess flavin and heme electron carriers responsible for transporting electrons across the membrane in order to reduce cytosolic oxygen into superoxide [Cross et al., 2004]. Still, NOX has a well established role in non pathological conditions, it generates ROS important for blood pressure maintenance in arteries and veins [Cifuentes & Pagano, 2006], Na^+ transport, tubuloglomerular feedback and renal oxygenation in kidneys [Wilkox, 2003, 2005; Zou & Cowley, 2003], Na^+/H^+ exchange in the loop of Henle [Junkos et al., 2006], airway and vascular remodelling in the lungs [Hoidal et al., 2003], smooth muscle cell proliferation and differentiation [Brar et al., 2002; Piao et al., 2005], gastrointestinal secretion and motility through serotonin production in the colon mucosa [Kojim et al., 2002], normal function of the central nervous system [Wang et al., 2004], osteoclast differentiation [Goettsch et al., 2013]. However, super-activation of NOX leads to excessive ROS production and this feature makes these enzymes not only exciting regulators of intracellular signalling but also potential drug targets.

Fifty years ago it was discovered that the electron transfer chain along the inner mitochondrial membrane is responsible of ROS production. Given that mitochondria utilize 95% of the oxygen assumed through inhalation, it was believed that they play a crucial role in cellular ROS production, although today the evidence for this statement is lacking. There are eight sites in mitochondria known to be able to produce $O_2^{-\cdot}$; while seven sites release $O_2^{-\cdot}$ into the mitochondrial matrix, only one site (complex III of the electron transport chain) releases it in the intermembrane space. Even if it is still unclear which site is physiologically the most important, it is believed that intermembrane ROS possess an advantage in accessing the cytosol more quickly respect to the matrix ones, as they have to cross only one mitochondrial membrane [Muller et al., 2004]. Moreover, mitochondria contain several other oxidoreductases which contribution to mitochondrial ROS production is still unknown.

In cytosol, xanthine oxidase system is the major ROS generator. It consists of two inter-convertible forms known as xanthine oxidase and xanthine dehydrogenase that catalyse conversion of hypoxanthine and xanthine to uric acid. Xanthine dehydrogenase functions in normal tissues and transfers electrons to

$NADP^+$. In damaged tissues, reversible oxidation of the cysteine residues or irreversible Ca^{2+}-proteolysis leads to transformation of dehydrogenase to oxidase, which transfers electrons directly to molecular oxygen [McCord et al, 1985]. Both processes eventually lead to ROS production.

Peroxisomes contain metabolizing enzymes from fatty acid α- and β-oxidation pathway, amino acid metabolism, synthesis of lipid compounds, xanthine oxidase and inducible form of nitric oxide synthase, that majorly contribute to the generation of moderately reactive and membrane-permeant ROS and RNS in this cellular site [Angermüller et al., 1987; Singh, 1997; Stolz et al., 2002]. Due to the abundance of metal ions like iron and copper in peroxisomes, under certain conditions these ions may be released and catalyse the formation of the most reactive ROS specie, $OH^{\cdot}$, thus leading to peroxisome damage and function alteration.

Lysosomes, due to their role in accumulation and enzymatic degradation of macromolecules and non-fragmented subcellular entities (autophagocytosis), require high proton concentration in order to provide an optimal pH for its metabolic enzymes. Therefore, as a support to this process, they contain electron transport system that gives rise to $OH^{\cdot}$ [Gille & Nohl, 2000]. The acid pH-milieu as well as the presence of iron represent favourable condition for spontaneous dismutation of $O_2^{-\cdot}$ into H_2O_2 and generation of $OH^{\cdot}$.

Multifunctional endoplasmic reticulum presents a variety of processes, but the ability to produce ROS is mainly represented by xenobiotic metabolism important for cell detoxification as xenobiotics are converted to more water-soluble and easily excreted species. The key role is played by a system called cytochrome P450 (CYP) that is the major producer of ROS in the liver. Endoplasmic reticulum presents another multienzyme system, called desaturase, devoted to introduction of double bonds in fatty acids. The two electron transfer systems collaborate in order to make the catalysis faster and reduce the time necessary for formation of side-products such as H_2O_2 and $O_2^{-\cdot}$ [Stadtman, 1986]. However, they can leak electrons and still represent an additional source of ROS *in vivo* [Schenkman & Jansson, 2003]. Moreover, in endoplasmic reticulum newly synthetized proteins undergo oxidative folding in order to stabilize their structure. Oxidized (GSSG) and reduced (GSH) forms of glutathione ensure formation of

disulfide bonds through oxidation of cysteine sulfhydryl groups.

The physiologically important RNS, NO·, is produced from the metabolism of L-arginine by nitric oxide synthases (NOS) whose three isoforms, neuronal (NOS1), endothelial (NOS3) and inducible (NOS2), have been identified. It has been reported that NOS1 and NOS3 are constitutively expressed, while NOS2 is induced by immune response. However, more recently, it has been shown that NOS2 is constitutively expressed in neurons, kidney, liver, lung, colon and keratinocytes [Villanueva & Giulivi, 2011]. This reactive specie plays different roles in different tissues, i.e. in macrophages contributes to their destroying action, in neurons act as a neurotransmitter and in blood vessels induces dilatation. The subcellular distribution of NOS is crucial for NO· signal transduction activities given its short half-life and limited diffusion by its interaction with different molecules within the cell [Cooper & Giulivi, 2007]. Modification of NOS isoforms may affect their subcellular compartmentalization, suggesting the occurrence of post-translational modifications such as phosphorylation or the activation of translocation, a complex process that requires specific protein-protein interactions, described for NOS3 [Schilling et al., 2006]. For example, in cardiac and endothelial cells, NOS3 is mainly found on plasma membranes, and in particular it is localized at the caveolae of the sarcolemma and T tubules. However, in response to different stimuli, NOS3 shuttles between caveolae and different intracellular sites [Sánchez et al., 2006].

Many ROS sources share an intricate redox-sensitive relationship and seem to cooperate in the maintenance of cellular ROS homeostasis. Therefore, given the complexity of interactions among various sources of ROS generation, it is not clear which site contributes most to physiological and pathological conditions ascribed to ROS [Camões et al., 2009; Vannuvel et al., 2013]. It has been reported that cyclooxygenase metabolites stimulate the generation of ROS by NOX [Hong et al., 2008], and that inside peroxisomes H_2O_2 and NO· are not circumscribed to their generation site due to their ability to cross the membrane [Fritz et al., 2007]. It has been demonstrated that mitochondrial-induced ROS can up-regulate xanthine oxidase [Gladden et al., 2011], and in a model of cocaine-induced diastolic dysfunction, it has been seen a crosstalk between cytosolic xanthine oxidase system and mitochondria respiratory chain [Vergeade et al, 2012]. Moreover, ROS produced in a limited number of mitochondria can influence neighbouring

mitochondria and other organelles, thus eventually propagating ROS to the whole cell.

For a long time before the breakthrough of ROS as physiological signalling mediators, they were thought to cause exclusively damaging effects. In the late '80s the terminology "oxidative stress" has been adopted after recognizing that the imbalance between oxidizing agents and antioxidant systems, in favour of the first ones, potentially led to damage [Sies, 1985, 1986, 1991]. Detrimental effects of ROS include DNA mutation, modification of gene expression, protein oxidation with consequent loss of sulfhydryl groups and modifications of amino acids that alter protein function, lipid peroxidation with eventual damage to cell membranes, all resulting in apoptosis or necrosis. In fact, oxidative stress is related to development of pathological conditions such as cardiovascular disease, type 2 diabetes, musculoskeletal disorders, rheumatoid arthritis, inflammatory bowel disease, cancer, neurodegenerative disease and many others (Fig. 3). In fact, ROS and RNS can have either beneficial or harming role for living systems. At low concentrations they act as signalling mediators being their function and specificity tightly regulated on several levels, whereas at high concentrations they cause damage to target macromolecules. Therefore, a steady balance in the production and removal of ROS is vital for the correct function of the intracellular processes.

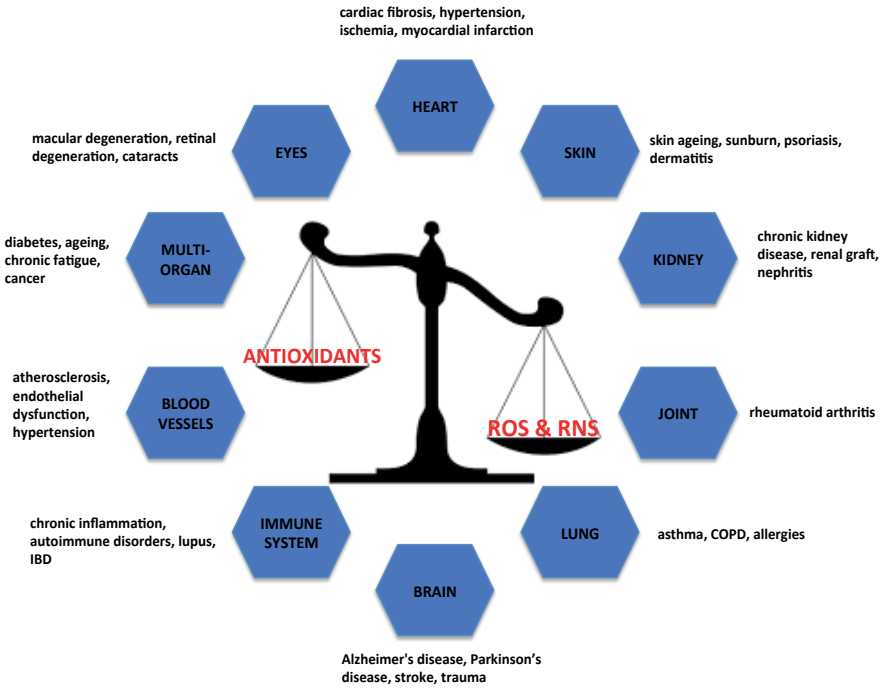


Figure 3. Effects of oxidative stress on human health.

2. Antioxidants control intracellular redox signalling

In general, any abnormal increase in oxidative stress-promoting substance (pro-oxidant) is mitigated by an antioxidant response. The term *antioxidant* originally was used to refer specifically to a chemical compound that prevented the consumption of oxygen. However, the identification of vitamins A, C and E as antioxidants led to realization of the importance of antioxidants in the biochemistry of living organisms [Knight, 1998]. Antioxidants remove free radicals and inhibit oxidation by being oxidized themselves. Alterations of pro-oxidant/antioxidant balance can induce oxidative stress and this is due to overproduction of reactive species and/or insufficiency of the antioxidant defence.

The antioxidant defence capacity and, therefore ROS signalling, are regulated at several levels divided in endogenous enzymatic and non-enzymatic antioxidants and exogenous antioxidants.

2.1. Endogenous intracellular antioxidants

Endogenous enzymatic antioxidant system is composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione reductase (GSR) and glutathione S-transferase (GST), thioredoxin reductase (TrxR), peroxiredoxins (Prx) and heme oxygenase (HO), whereas endogenous non-enzymatic antioxidant system is composed of glutathione (GSH), thiorexodin (Trx) and melatonin. Nevertheless, among these antioxidant regulators, two major systems are responsible for maintaining an intracellular reduced state: thioredoxin and glutathione systems.

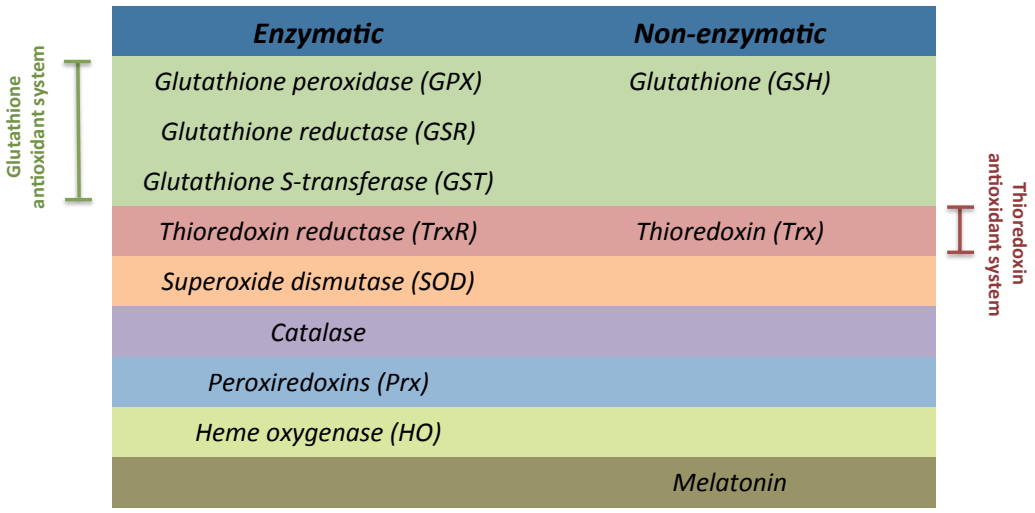


Table 1. Endogenous intracellular antioxidants.

Glutathione antioxidant system is formed by three enzymes, GPX, GSR and GST. GSH is a tripeptide also known as L-γ-glutamyl-L-cysteinyl-glicine, important for the maintenance of optimal intracellular redox state thus preserving the integrity of cellular proteins and lipids. Its functions depend on the redox-active thiol of the cysteine moiety that serves as a cofactor for a number of antioxidant and detoxifying enzymes. Reduced GSH is the biologically active form that is oxidized to glutathione disulfide (GSSG) during oxidative stress; GSH/GSSG ratio thus offers a simple and convenient marker of cellular oxidative stress. Sources of GSH are cytosolic *de novo* synthesis from its constituent amino acids, enzymatic reduction of GSSH by GSR, and exogenous uptake. Its specificity and targeted redox control are achieved through unique compartmentalization of GSH. In fact, it is found in cytosol, mitochondria, endoplasmic reticulum and nucleus. Cytosolic GSH ranges between 2-10 mM in most cell types, whereas GSH/GSSG ratio under physiological conditions is very high, ie. 100/1 in liver, while it decreases during oxidative stress and apoptosis. Mitochondrial matrix and endoplasmic reticulum GSH levels equal that of cytosol, whereas nuclear GSH levels seem to be lower as their import occurs by passive diffusion via nuclear pores. The absence of concentration gradient and the presence of negative charge at physiological pH value, require specific carriers for GSH transport from cytosol to mitochondria. Moreover, as mitochondria play a strategic role in activation of cell death pathways, including apoptosis and necrosis, mitochondrial GSH arises as regulator

of susceptibility and outcome of cell death. For this reason, it is important to replenish mitochondrial GSH pool in order to counteract mitochondrial oxidative stress, a central event of many pathological conditions, especially when GSH transport may seem to be defective due to alterations in membrane dynamics caused by apoptotic stimuli. Mitochondrial-permeable GSH-ethyl ester is able to bypass this defect, and S-D-lactoylglutathione is another compound able to be hydrolysed in the matrix thus yielding GSH along with lactate. On the other hand, direct cytosolic GSH boosters, i.e. N-acetylcysteine (NAC), are not indicated for this purpose as they increase only cytosolic GSH that has no chance to enter mitochondria.

GPX contains four selenium-cofactors that catalyse the oxidation of GSH to GSSG, and the reduction of H_2O_2 to H_2O and lipid hydro-peroxides to corresponding stable alcohols. There are at least four different GPX isoenzymes among which the most abundant and the most active is GPX1. Particularly high levels are found in the liver and serve in detoxification metabolism. GPX reaction is coupled with GSR that regenerates reduced form of GSH.

GSR catalyses the NADPH-driven reduction of GSSG to GSH. Its function is to maintain the intracellular concentration of GSH high and that of GSSG low.

GST, also known as glutathione S-transferase, represents a major cellular defence system. It belongs to a superfamily of proteins divided in seven families (Alpha, Mu, Pi, Theta, Sigma, Zeta and Omega), ubiquitously expressed in most living organisms. It has been shown that these enzymes catalyse reactions ranging from detoxification to biosynthesis and cell signalling. They are involved in metabolic processes of endogenous lipid mediators, such as prostaglandins [Beuckmann et al., 2000], steroids [Johansson et al., 2001], leukotrienes [Anuradha et al., 2000], and in the regulation of toxic products generated by oxidative stress [Alin et al., 1985; Listowsky et al., 2005]. GSTs comprise three families of proteins: cytosolic, mitochondrial and microsomal. They show a high degree of polymorphism and include different subunits. Each subunit (consisting of about 199-244 amino acids and weight of 22-29 kDa) contains an active, catalytically independent site in the N-terminal domain to which GSH binds (G site), and a site in C-terminal domain to which hydrophobic substrate binds (H site).

More recently, it has been shown that several GST isoenzymes modulate cellular

signalling pathways by forming protein-protein interactions with critical proteins involved in controlling stress response, apoptosis and proliferation [Miraoui et al., 2009; Ge et al., 2012]. The GSTPi may be the most peculiar GST having an inhibitory role in various signalling pathways implicated in apoptosis or proliferation. Among the proteins with which GSTP interacts there is c/Jun N/terminal kinase (JNK), which in particular binds to the GSTP1-1 isoenzyme [McIlwain et al., 2005]. Adler et al. were the first in demonstrating that mouse GSTPi forms a complex with JNK. Dissociation of this complex by different types of factors, such as oxidative stress, leads to the activation of JNK and phosphorylation of its substrate, the transcription factor c-jun. Another function of GSTPi involves post-translational modification of proteins, the S-glutathionylation, and thus protection against oxidative damage and control of redox signalling. Pedersen et al. have brought up a new insight on NO stockage by GSTP1-1. In particular, GSTP1-1 binds NO present as dinitrosyl iron complexes (DNICs), and this seems to protect cells against high levels of DNICs that can inhibit GSR leading to GSH depletion. This could also represent a key signal to trigger apoptosis.

Recently, it has been shown that in basal conditions, i.e. in the absence of cytotoxic stimuli, the monomeric form of GSTP1-1 binds to the C-terminus of JNK, keeping this kinase inactive. In this way, JNK intracellular signalling pathway and, therefore, apoptotic signal is inhibited. Oxidative stress and increased ROS levels cause GSTP1-1 dissociation from JNK resulting in kinase activation and induction of apoptotic processes (Fig. 4) [Adler et al., 1999]. Several studies have led to the identification of a protein complex consisting of GSTP1-1/JNK/c-Jun that selectively inhibits c-Jun phosphorylation by JNK. An increased activity of JNK was highlighted following UV irradiation or H_2O_2 treatment, resulting in dissociation of the monomeric form of GSTP1-1 from JNK. When GSTP1-1 is dissociated from JNK, it oligomerizes giving rise to a high-molecular weight form of 97 kDa. In this condition JNK is active and increases apoptosis. From this it emerges that GSTP1-1 inhibits JNK by forming a protein complex that dissociates in conditions such as oxidative stress, which induce the oligomerization of GSTP1-1 [Adler et al., 1999]. In fact, ROS determine dissociation of GSTP1-1 from JNK in order to maintain the equilibrium between the monomeric and the oligomeric forms of GSTP1-1. In the presence of ROS, there is an increase of GSTP1-1 oligomeric form with respect to

the monomeric one, which is then dissociated from JNK. The formation of disulfide bond between the Cys-47 of GSTP1-1 monomers is responsible of inhibition of GST activity, which could be explained by the localization of these cysteines in the GSH-binding domain. Because of the overexpression of GSTP1-1 in several tumors, as well as of its dual role as an enzyme involved in both deactivation of antitumoral agents and inhibition of signalling pathways leading to apoptosis, GSTP1-1 can be considered a good therapeutic target in cancer therapy.

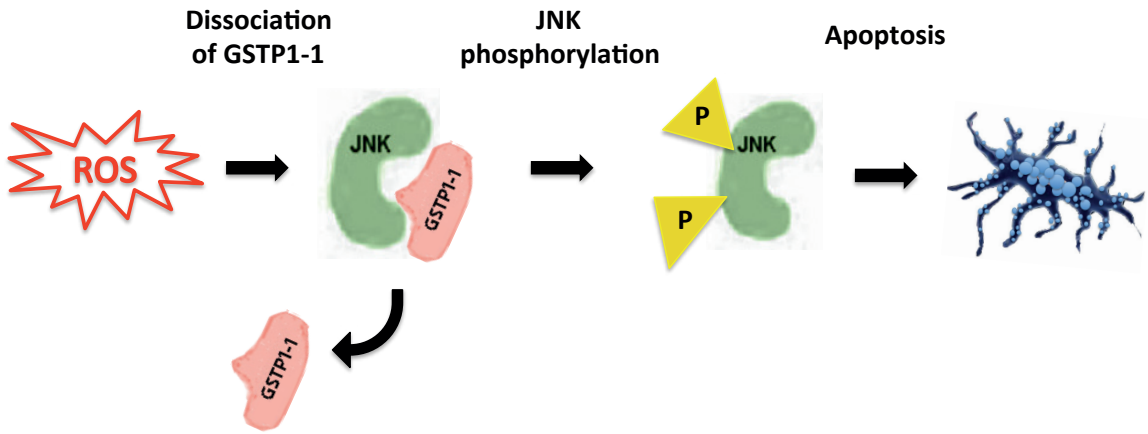


Figure 4. GSTP1-1 role in intracellular signalling.

Thioredoxin antioxidant system is a pivotal partner with glutathione redox system in redox regulation, and is composed of Trx and TrxR. Trx is disulfide-containing oxidoreductase that modulates the activity of redox-sensitive transcription factors. It is present in cytoplasm, mitochondria, on cellular membrane and in extracellular space. Oxidized cysteines in Trx active site are reduced by TrxR and NADPH to active dithiols which are responsible for ROS scavenging. Trx negative regulator is a thioredoxin binding protein that exerts multiple regulatory functions regarding cellular redox state, growth, aging and apoptosis. In fact, Trx regulation is distinct in various cell compartments as well as independent of GSH/GSSG regulation, and generally represents a crucial mechanism in the regulation of redox-sensitive biological processes.

Prx are a group of non-seleno thiol-specific peroxidases that also contribute to cellular redox homeostasis. Prx are localized in cytosol, mitochondria, peroxisomes and in the extracellular space. Within their catalytic site they possess reactive cysteines that are oxidized to sulfenic acids (Cys-SOH) and rapidly form disulfide bonds with another cysteine at the C-terminal unit. These enzymes rely on thioredoxin system as cysteine regeneration is catalysed by TrxR.

SODs are metal ion cofactor-requiring enzymes that provide major antioxidant defences against ROS by catalysing dismutation of $O_2^{-\cdot}$ into O_2 and H_2O_2. There are three isoforms of SOD in humans: cytosolic (Cu-Zn-SOD), mitochondrial (Mn-SOD) and extracellular Cu-Zn-containing SOD (EC-SOD). The first one is a dimer while others are tetramers. Their levels change altogether or separately upon cell damage depending on the tissue involved. In particular, Nojiri et al. have demonstrated that cytoplasmic SOD plays a pivotal role in the development and progression of bone fragility during aging, leading to decreased BMD, low-turnover osteoporosis and impaired collagen cross-linking. In inflammatory bowel disease (IBD) patient's epithelium, all the three SOD isoforms are up-regulated [Kruidenier et al., 2003], while colorectal cancer is associated with enhanced mitochondrial SOD expression.

Catalases are heme enzymes prevalently found in peroxisomes that dismutate H_2O_2 to H_2O and O_2. Particularly high concentrations of this enzyme occur in liver, kidneys and enterocytes due to the necessity for rapid decomposition of harmful H_2O_2. Some scientists claim that their main function is to protect cells from apoptosis [Neonoi et al., 2001].

HO comprises two isoforms, HO1 inducible and HO2 constitutive, that catalyse degradation of heme generating CO, biliverdin and iron. HO1 does not have a direct antioxidant enzymatic function but rather an indirect cytoprotective response against oxidative stress and apoptosis, given that oxidative stress is able to strongly induce HO1 expression.

Melatonin is an ubiquitous hormone primary synthetized in mammalian pineal gland. As an antioxidant is irreversibly oxidized and cannot be reduced, and thus is referred to as a "suicidal antioxidant". During oxidation is converted to several antioxidant intermediate metabolites found in nuclei and mitochondria, as melatonin is able to cross the membranes.

2.2. Exogenous antioxidants and their double-edged effect

As previously seen, some antioxidants are constitutive and/or produced during normal metabolism, while other antioxidants are of exogenous origin. Both, endogenous and exogenous antioxidant defence systems are incomplete one without another. Therefore, there is a continuous demand for exogenous antioxidants in order to prevent oxidative stress and related diseases. For example, ascorbic acid, vitamin E, CoQ_{10}, carotenoids and polyphenols are obtained from external sources and play an important role in maintaining human health. Dietary intake is a very important source of natural antioxidants and micronutrients, and points to the potential effects of malnutrition or malabsorption of nutrients on the regulation of these mediators. Synthetic and natural food antioxidants are routinely used in food industry, cosmetics and pharmaceutical industry to protect foods or medicine, especially those containing oils and fats, against oxidation. Moreover, in view of increasing factors for various diseases, there has been a global trend towards the use of natural substances as prophylactic or therapeutic agents in many diseases. The interest in natural antioxidants is determined by the universality of their action in various redox systems and consequently broad spectra of possible applications. There are several reports showing antioxidant potential of fruits, whereas tea plants have been extensively studied in the recent past for antioxidant properties since they contain phenolic compounds. Epidemiological studies are the main contributors in enlightening the potentials of these agents in prophylaxis and therapy. They have indicated a significant difference in the incidence of various diseases among ethnic groups with different lifestyles and exposure to different environmental factors. The best examples to prove the efficiency of dietary antioxidants are given by French paradox and

Mediterranean diet. French paradox is about the low cardiovascular mortality in spite of high-fat intake in French population due to consumption of red wine rich in antioxidants such as resveratrol. Mediterranean diet is also rich in antioxidants deriving from fruits, vegetables, whole grain, legumes and olive oil. However, many of the benefits derived from intake of dietary antioxidants are the result of synergism between natural antioxidants, vitamins and endogenous antioxidant defence system.

Oral bioavailability of natural antioxidants, however, depend on numerous factors such as food processing, food deprivation, stability of the antioxidant, stabilizing effect of food matrix towards the release of lipophilic antioxidants, possible presence of isomeric form, the presence of glycosylated form in case of polyphenols that influence their solubility and partition coefficient, hydrolytic capacity of the intestine towards conjugated sugar moiety. Therefore, the necessity for formulated antioxidant drugs over dietary antioxidants emerged over time. Formulated antioxidant drugs are generally administered as concentrated agents in a pharmacological formulation, thus being available at higher concentrations and rapidly saturating metabolic pathways that metabolize them. Large doses are metabolized primarily in the liver, while small doses may be metabolized in intestinal mucosa and microbial flora. However, oral bioavailability problems might occur even with conventional antioxidant formulations. These regard some physicochemical and biopharmaceutical properties such as solubility, permeability, stability and, if necessary, biotransformation. Therefore, sometimes is necessary to design non-conventional targeted drug delivery systems in order to have a successful delivery of antioxidants and maximize their role in prophylaxis and therapy.

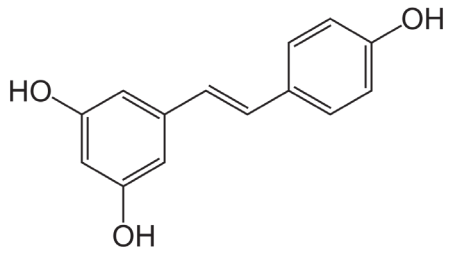
Moreover, it has to be taken in consideration that high doses of these compounds may be toxic due to their pro-oxidative effects or interference with beneficial concentrations of ROS normally present in physiological conditions. In addition to the concentration of antioxidants, the presence of metal ions, especially Fe^{3+} and Cu^{2+}, has been reported to chelate phenolic compounds [Raza & John, 2005]. In this regard, it has been shown that the pH influences oxidoreductions of phenolic compounds, suggesting that pH of biological tissues could impact antioxidant or pro-oxidant activities of phenolic compounds and their chelating

activity. It is worth mentioning that beneficial or harmful effects of natural compounds may also occur independently from their (anti)-oxidative properties as a result of the activation of particular cellular pathways. Therefore, physiological doses of exogenous antioxidants are important in order to maintain or re-establish redox homeostasis [Ratnam et al, 2006].

Apart form natural antioxidants, their synthetic derivatives represent a valid substituent when it comes to overcoming absorption, metabolism and bioavailability problems that limit the effect of natural antioxidants. A large amount of effort is needed to design compounds with improved efficiency, low toxicity and minimum side effects. Firstly, biological activity and molecular mechanism of a certain natural antioxidant are investigated. Then structural modifications are made to help understanding the structure-activity relationships of biological activity of antioxidants. Finally, different analogues are synthetized and their biological effects are compared. However, molecular mechanisms are not studied sometimes in the initial phase and the "black box" approach is employed to find compounds that are efficient in modulating redox state. There are numerous examples of successful use of natural antioxidant derivatives to ameliorate pathologic events due to oxidative stress. For example, some selenium derivatives of vitamin E have received great attention due to their wide therapeutic windows and minimally toxic effects on normal cells. These modifications have led to intermediates and metabolites able to generate desired and efficient signal [Vraka et al. 2006].

2.3. Antioxidants used in this study

a) Natural antioxidants



Resveratrol (RE) (3,4',5-trihyroxystilbene) belongs to a class of polyphenolic compounds called stilbenes. Certain plants produce RE in response to different types of ambient stress. It occurs in both *trans* and *cis* molecular configurations, but *trans*-RE appears to be well absorbed in humans when taken orally. However, its bioavailability is relatively low due to its rapid metabolism and elimination. Once absorbed it is rapidly metabolized by conjugation to glucuronic acid and sulfate, thus forming glucuronides, sulfates or sulfoglucuronides [Walle, 2011]. Moreover, RE bioavailability shows a high inter-individual variability due to gut microbiota influence on its metabolism. RE-containing liquid formulations are easier to absorb respect to capsules, which, on the contrary, make sure that RE metabolites remain longer in the organism. Food has been reported to delay RE absorption but not its bioavailability. Preclinical studies have shown that RE possesses numerous biological activities, such as direct antioxidant activity, estrogenic and anti-estrogenic activity depending on: cell type, estrogen receptor isoform and the presence of endogenous estrogens [Tangkeangsirisin & Serrero, 2005]. Moreover, recently RE was shown to improve endothelial would healing through estrogen receptor-dependent pathway in an animal model of arterial injury [Yurdagul et al., 2014]. RE has been found to exert beneficial effects against metabolic and immune-mediated pathologies, as well as in the prevention of cardiovascular diseases, neurodegenerative diseases, cancer, in the improvement of glycemic control of type 2 diabetes, inflammation and aging. Beneficial effects of RE are also ascribed to its antioxidant activity given its ability to boost intracellular antioxidant enzymes, prevent low density lipoprotein (LDL) oxidation and scavenge ROS. RE is able to induce the expression of antioxidant genes, increase the activity of antioxidant enzymes and reduce the content of oxidized LDL induced by ROS increase in cardiovascular, musculoskeletal and nervous system, as well as in hepatocytes [Liu et al., 2004, 2012; Rubiolo et al., 2008; Inglés et al.,

2014]. Its antioxidant activity has also been determined in isolated rat brain mitochondria, where RE inhibited the activity of complex III by competing with coenzyme Q [Zini et al., 1999].

RE is found in grapes, wine, grape juice, Itadori tea (obtained from *Fallopia japonica*, traditional herbal remedy for heart disease and strokes), peanuts, cocoa and berries of *Vaccinium* species, such as blueberries, bilberries and cranberries (Fig. 5).

Figure 4. Resveratrol natural sources.

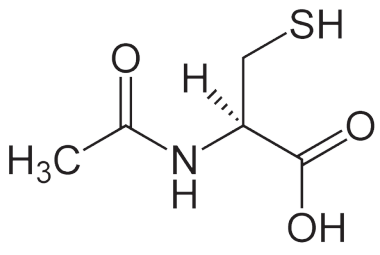
In grapes, RE is found only in the skin and the amount of RE varies in relationship of grape cultivar, geographic origin and exposure to fungal infection. Moreover, the amount of time that grape skins spend in contact with wine during fermentation is also important factor for RE content. In fact, in the production process of white and rosé wines grape skins are being removed resulting in less RE. However, it results that RE is not the predominant polyphenol in red wines and that its concentration is rather low. Adverse effects are still unknown, however, one follow-up study has shown minor adverse effects such as nausea, flatulence, diarrhea in patience that were given more than 1000 mg/die RE for 29 consecutive days. Moreover, high dosages of RE inhibited CYPs isoenzymes that are critically involved in the bioavailability, metabolism, activation and detoxification of many xenobiotics. For example, RE inhibits CYP3A4 important for statin bioavailability. Statins are lipid-lowering drugs of primary choice in the prevention of cardiovascular diseases. High doses of RE could alter the metabolism of statins provoking serious adverse effects [Chow et al., 2010].

Blueberry extract (BE) is a methanolic dry preparation deriving from fresh fruits of edible berry species such as *Vaccinium myrtillus* commonly known as European blueberry. It is found natively in Europe, northern Asia, Western United States, Western Canada and Greenland. Blueberries (BB) are fruits with high biological activity due to their antioxidant compounds such as polyphenols, proanthocyanidins and anthocyanosides. Anthocyanic compounds are present in the form of glycosides, antocyanins. The most important anthocyanins are the cyanidin glycosides, as they represent 50% of the pigment composition of fruits [Kong et al., 2003]. Fourteen anthocyanins have been identified in blueberry fruit, juice and extract, and their amount increases as fruits ripen. Moreover, there is a great diversity of anthocyanin content in various geographical areas. Their stability is affected by various factors (high temperature, basic pH, enzymes, UV, ascorbic acid, metal ions) resulting in colour change and degradation. Although BBs are promoted most commonly for improving vision, vasoactive properties, it has been reported that BBs also lower blood glucose, have anti-inflammatory and lipid-lowering effects, promote antioxidant defence and lower oxidative stress. They safely improve osteoporosis condition in a rat model of ovariectomy-induced osteoporosis, which is the most common cause of age-related bone loss [Li et al. 2014]. It has been demonstrated that 10% BB-supplemented diet promotes osteoblastic bone formation due to phenolic acid metabolites deriving from polyphenols present in BB [Chen et al., 2010], and that it also suppresses bone resorption through RANKL down-regulation in bone marrow stromal cells [Zhang et al., 2013]. Moreover, BB diet, preferably along physical activity, results important for critical bone-forming years between the ages of 12 and 18 years, delaying greatly the onset of osteoporosis due to aging-process [Novotny et al., 2004; Gilsanz & Wren, 2007; Zhang et al., 2011].

Green tea extract (GTE) is a concentrated supplemental form of green tea (GT) obtained through extraction process using ethanol as a solvent. GT is one of the most popular beverages in the world. It has legendary origins from China and derives from dried leaves of *Camellia sinensis* that have not undergone oxidation process typical for black tea. This makes it rich in polyphenolic components (up to 30% dry weight), also called catechins or tea polyphenols, with strong antioxidant and anti-inflammatory activity. These compounds mainly include (-)-epigallocatechin gallate, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epicatechin, that also contribute to stabilization of GTE. Catechins are hypothesized to help against oxidative damage by contributing directly or indirectly, along with antioxidant vitamins (C and E) and enzymes, respectively, to the total antioxidant defence system [Abdrel-Raheim et al., 2009]. In addition to this, GT effectiveness is determined by the rate of reaction of its catechins with free radicals. It has also been reported that tea polyphenols may also inhibit xanthine oxidase [Higdon & Frei, 2003]. These results have been confirmed *in vivo* in many studies, while clinical studies result more complex. The association between GT consumption and human health has long been appreciated. Numerous *in vitro* and *in vivo* studies have suggested the beneficial health properties of GT and its polyphenols including antioxidant and antitumor effects, hypertension and hyperlipidemia reduction and antimicrobial activity. Various studies show that green tea drinking/ingesting may mitigate age-related bone loss in elderly men and women [Shen et al., 2009] as well as chronic inflammation-induced bone loss [Shen et al., 2010]. Green tea components inhibit formation and differentiation of osteoclasts, block RANKL signalling essential for osteoclastogenesis and induce osteoclast apoptosis [Nakagawa et al., 2002; Hafeez et al., 2006; Lin et al., 2009a; Oka et al., 2012]. Moreover, green tea stimulates osteoblastogenesis and bone mineralization [Chen et al., 2005; Vali et al., 2007].

Hypericum perforatum extract (HE) derives from a medicinal herb and a popular folk remedy widely used for treatment of various disorders. It grows in Europe, Northern Africa, Northern America and in some China provinces. It has been demonstrated that HE contains several classes of plant phenolic compounds with anti-depressive activity due to inhibition of serotonin reuptake, as well as anti-inflammatory activity due to its flavonoid components with estrogen-mimetic properties in addition to antiviral, antibacterial and antioxidant activity. Although several publications have been focused on HE antioxidant properties, there is still a lack of conclusive evidence to determine which class is the most responsible for that. Orčić et al., by using fractionation combined with assays and LC-MS analysis, have shown that mostly flavonoid glycosides and phenolic acids had the antioxidant activity. Zou et al. have demonstrated that flavonoid-rich extract, prepared through HPLC, exhibited strong antioxidant activity, prevented the peroxidation of lipid membranes in liposome and acted as effective $O_2^{\cdot-}$ scavenger.
It has been shown that *Hypericum perforatum* (HP) has beneficial effects on osteoblast proliferation and estrogen deficiency-related bone loss in postmenopausal women [You et al., 2015].

b) Synthetic antioxidants



NAC is a thiol compound, precursor of GSH. For more than thirty years it has been in clinical use as a mucolytic, and it is studied and utilized in conditions characterized by decreased GSH state during oxidative stress. Because of its hepato-protective activity, taken orally or intravenously it is used as the antidote in acetaminophen poisoning. Once absorbed rapidly, NAC goes through extensive first pass metabolism by small intestine cells ad the liver. Only a small percentage of intact NAC reaches the plasma. However, its apparent low bioavailability is misleading

due to the fact that in the metabolic processing of NAC a variety of beneficial SH-containing metabolites are formed. The sulfhydryl group is majorly responsible for NAC metabolic activity, while acetylation makes NAC more stable against oxidation. NAC mechanisms of action regard the reduction of extracellular cystine to cysteine as well as generation of SH metabolites by which NAC can stimulate GSH synthesis, enhance GST activity, promote detoxification and act as a direct ROS scavenger. Moreover, NAC appears to enhance GSH synthesis only in conditions when it is low during oxidative stress. In fact, NAC is a highly effective component of a nutritional supplementation protocol in conditions of excessive oxidative stress that characterizes chronic exposure to cigarette smoke and heart disease. Other successful clinical implications of NAC regard acute respiratory distress syndrome, chronic bronchitis, cancer, epilepsy, HIV infection, Sjogren's syndrome, heavy metal poisoning and influenza prevention [Kelly, 1998].

RE synthetic benzoselenophene derivatives have been developed recently by Tanini et al., as findings on RE poor bioavailability have always been a classical drawback for this molecule, although its effects indeed exist. Moreover, structural modifications of RE are useful for ameliorating some of its biological effects. Selenium is an essential micronutrient important for the appropriate functioning of a number of selenium-dependent enzymes constituting glutathione and thioredoxin antioxidant systems. Twenty-five genes coding for selenoproteins have been identified in humans, thus suggesting that the insertion of selenium in the enzyme structure, in particular in the catalytic site, is important for the redox activity of the enzyme. In organic chemistry, selenium-based functionalization of phenolic compounds has been applied to improve their antioxidant effect due to decrease of the bond dissociation enthalpy of phenolic groups. Three benzoselenophene derivatives with increased rigidity have been synthesized. These compounds are named VD0, VD1 and VD2 depending on the number of chlorine atoms present in their structure (Fig. 6).

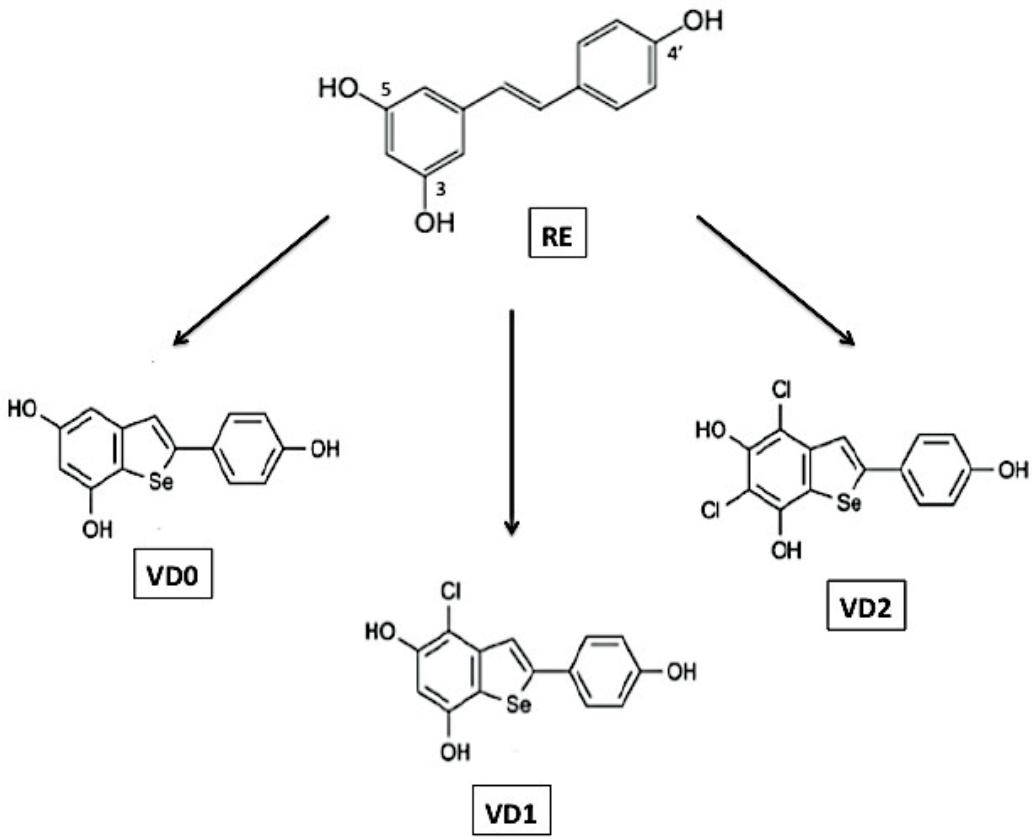


Figure 6. Structure of RE and its benzoselenophene derivatives VD0, VD1 and VD2.

3. Bone remodelling

3.1. Bone remodelling and factors related to bone absorption and mineralization

Bone and cartilage constitute the skeletal system, which serves two main functions. The first is a structural function that consists in support and protection of internal organs and bone marrow, as well as in the attachment for muscles important for locomotion. The second is a metabolic function serving as a reserve of calcium and phosphate needed for the maintenance of serum homeostasis. At microscopic level, bone is a dynamic tissue continuously renewed through the process of bone remodelling firstly defined by Frost in 1990 [Frost, 1990; Cohen, 2006; Zaidi, 2007]. This process allows substitution of primary or infantile bone with mechanically more resistant secondary bone, the removal and substitution of damaged bone and the correct calcium and phosphate homeostasis. Bone remodelling requires the interaction between different cell phenotypes and is regulated by a variety of biochemical and mechanical factors. In fact, it results from the coupling action of bone forming cells –osteoblasts, and bone resorbing cells – osteoclasts. These cells closely collaborate in a specialized vascular entity called basic multicellular unit or bone remodelling compartment, which provides the structural basis for coupling and regulation of cellular activity.

The bone remodelling cycle begins with the activation mediated by the cells of the osteoblast lineage, and may involve their terminally differentiated cells – osteocytes, the lining cells that cover non-remodelling bone surfaces, and the preosteoblasts from the bone marrow. The activation signal can take several forms, e.g. direct mechanical strain on the bone that results in structural damage or hormone action on bone cells. Osteocytes sense changes in these physical forces and translate them into biological signals that initiate bone remodelling. Osteoblasts respond to signals generated by osteocytes or to direct endocrine signals, and produce chemoattractant substances that recruit osteoclast precursors to the remodelling site. In addition to this, osteoblasts express the master osteoclastogenesis cytokines such as colony stimulating factor 1 (CSF-1), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin

(OPG). While OPG expression is reduced being it a decoy receptor for RANKL, CSF-1 and RANKL expression are increased to promote osteoclast formation and subsequent activity. CSF-1 promotes proliferation and survival of osteoclast precursors and directs spreading, motility and cytoskeletal organisation in mature osteoclasts. RANKL also promotes proliferation of osteoclast precursors and coordinates the differentiation of osteoclast precursors to multinucleated osteoclasts, promotes resorption activity and prolongs the lifespan of mature cells. Moreover, osteoblasts secrete matrix metalloproteinase MMP-13 that degrades the unmineralized osteoid that lines the bone surface and expose mineralized bone sites necessary to facilitate osteoclast attachment. Once attached via integrin molecules, osteoclasts create an isolated microenvironment in which hydrogen ions H^+ are pumped causing acidification and dissolution of mineralized matrix thus forming Howship's lacunae. The remaining organic bone is then degraded by a variety of collagenolytic enzymes such as cathepsin K. Bone resorption takes from 2 to 4 weeks during each remodelling cycle. Completed their action, osteoclasts undergo apoptosis. Debris of undigested demineralized collagen matrix are removed by so-called reversal cells, thought to belong to osteoblastic lineage [Andresen et al., 2013]. These cells are located between osteoclasts and osteoblasts (Fig. 7), and this feature makes them obvious contributors to the osteoclast-osteoblast coupling [Delaisse, 2014] in order to allow transition from bone resorption to bone formation. Reversal cells gradually mature to osteoblasts and render eroded surfaces amenable to bone formation [Abdelgawad et al., 2016].

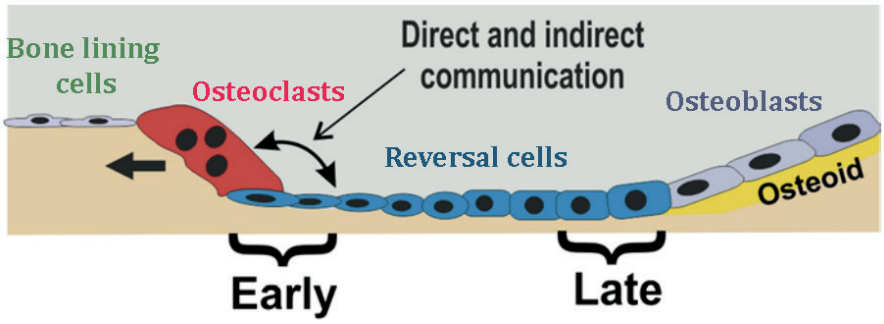


Figure 7. Model summarizing the main characteristics and functions of reversal cells. The early reversal cells have gradually matured from the immature preosteoblastic reversal cells into the late osteoblastic precursors prior to becoming mature osteoblasts.

It has been proposed that osteoclasts produce coupling factors, such as soluble molecule sphingosine 1-phosphate and cell-anchored EphB4-ephrinB2 bidirectional signalling complex [Martin & Sims, 2005]. Sphingosine 1-phosphate induces osteoblast recruitment and promotes mature osteoblast survival, while EphB4-ephrinB2 is able to simultaneously enhance osteoblast and supress osteoclast differentiation. Osteocytes also can exert bone formation signals. These cells express sclerostin, a soluble molecule produced from Sost gene that acts in a paracrine manner to inhibit bone formation by binding to LRP5 and LRP6 (low-density lipoprotein receptor-related protein-5/6) and thereby directly antagonizes Wnt signalling, an inducer of bone formation [Li et al., 2005]. Mechanical and endocrine stimulation through parathyroid hormone (PTH) inhibit osteocyte expression of sclerostin, removing the inhibition from Wnt signalling and allowing bone formation [Keller & Kneissel, 2005]. Once osteoblast progenitors have returned to resorption lacunae, they differentiate and secrete collagen I, proteoglycans, alkaline phosphatase, small integrin-biding ligand proteins, matrix Gla-protein, osteocalcin and lipids [Robey & Boskey, 2008]. Hydroxyapatite is incorporated into the newly deposited osteoid in order to give the bone its final form. Bone formation is completed in 4 to 6 months. When equal quantity of resorbed bone has been replaced, the remodelling cycle concludes (Fig. 8). The termination signal may be due to osteocyte sclerostin expression. At the completion of the cycle, a great deal of osteoblasts (50-70%) undergoes apoptosis and transforms into osteocytes or bone-lining cells.

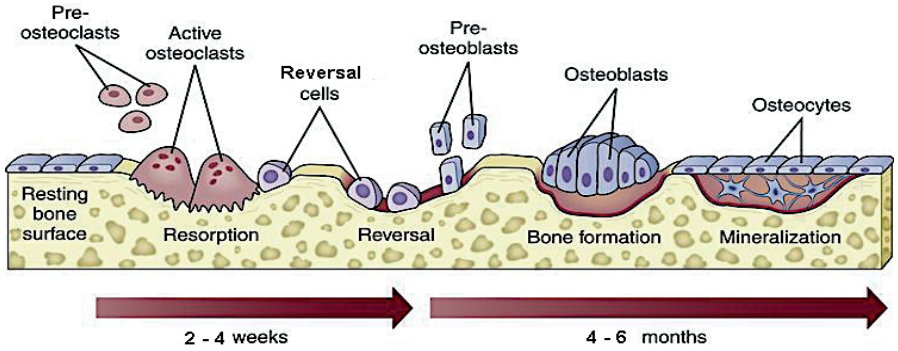


Figure 8. Schematic representation of a basic multicellular unit illustrating five stages of bone remodelling cycle: resting, resorption, reversal, formation and mineralisation phase.

There are both systemic and local regulatory factors acting on bone remodelling process. Systemic factors include PTH, calcitriol, calcitonin, glucocorticoids, thyroid hormones, estrogens and androgens, whereas local factors include OPG/RANKL/RANK system, tumor necrosis factor alpha (TNFα), interleukin (IL) -10 and -6.

PTH, a hormone secreted by parathyroid glands, regulates calcium homeostasis by stimulating Ca^{2+} release from bones or Ca^{2+} resorption from ultrafiltrate in kidneys. PTH has a biphasic effect on bone formation. PTH stimulates osteoclast formation by binding to its receptor, PTH receptor 1, on stromal-osteoblastic cells and thereby increases RANKL production and suppresses OPG decoy activity [Liu et al., 1998; Lee & Lorenzo, 1999]. Intermittent administration of PTH as a therapy to induce bone anabolism increases bone formation, while chronic PTH elevation (as in hyperthyroidism) increases bone resorption [O'Brien et al., 2005; Jilka, 2007]. The reason of this behaviour is still unknown. With the age, plasma levels of PTH tend to increase which may cause increment of bone turnover and loss of bone mass.

Calcitriol or vitamin D3, is the hormonally active metabolite of vitamin D produced in the proximal tubule cells of the nephron in the kidneys. The activity of the enzymes responsible for calcitriol production is controlled by PTH. Calcitriol stimulates intestinal absorption of calcium in order to make it available for bone mineralization. However, it can stimulate both bone formation and resorption, regulating bone turnover by acting on both osteoclastic and osteoblastic cell lineages.

Calcitonin is produced by parafollicular thyroid cells or C cells, and inhibits the function of osteoclasts either in physiologic and in pharmacologic doses. This effect is dose limited, and its physiologic role is minimal in adult skeleton.

Glucocorticoids exert both stimulatory and inhibitory effects on bone cells. They are essential for osteoblast maturation, but at the same time they also decrease osteoblast activity. Furthermore, they make bone cells more sensitive to regulators of bone remodelling and they increment osteoclast recruitment.

Thyroid hormones stimulate both bone formation and bone resorption, thus bone turnover results increased in hyperthyroidism and therefore bone loss can occur.

Estrogens are a group of sex hormones not only important for sexual development, but also for normal bone turnover *(see the section below).*

Androgens are essential for skeletal growth and maintenance via their effect on androgen receptor present in all types of bone cells.

Hormonal signals are modified and integrated with various environmental factors in different bone compartments, including mechanical strain, paracrine cytokines and growth factors. Local growth factors and cytokines come either from the cells in the marrow space or vascular cells having free access to the remodelling site without barriers, or are produced by osteoclasts and osteoblasts present at the remodelling site.

The OPG/RANKL/RANK system controls osteoclastogenesis and bone remodelling in general (Fig. 9). RANKL is a crucial factor for osteoclastogenesis. It belongs to the TNF family and is expressed on the surface of preosteoblastic cells, osteocytes and activated T cells. RANKL binds to receptor activator of nuclear factor kappa-B (RANK) expressed on the osteoclastic precursor cells, and this event is critical for differentiation, fusion into multinucleated cells, activation and survival of osteoclastic cells. This entire system is blocked by OPG binding to RANKL. The opposite phenotypes of OPG overexpression or with RANKL-deletion mice, and RANKL overexpression or with OPG-deficient mice, have led to the hypothesis that these two factors can be the mediators of stimulatory or inhibitory effects of other local factors on bone remodelling. This refers to as “the convergence hypothesis” in which the activity of resorptive and anti-resorptive agents converges to OPG and RANKL activity. Their ratio is therefore the marker of the degree of osteoclastogenesis.

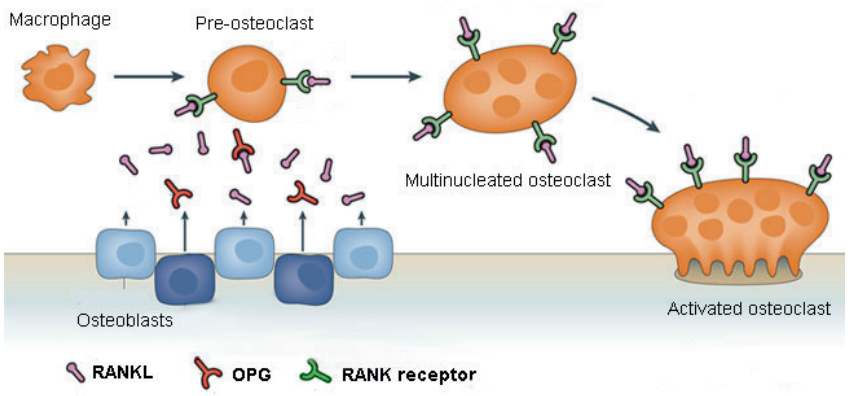


Figure 9. OPG/RANKL/RANK system

Cytokines such as TNFα and IL-10 can directly increase RANKL expression, and IL-6 stimulates osteoclastic bone resorption but also promotes generation of osteoblasts in conditions of high bone turnover [Moonga et al., 2002; Sims et al., 2004]. Bone also contains a large number of growth factors out of which the most abundant are insulin-like growth factors (IGFs), which act in local bone remodelling. Other growth factors such as transforming growth factor beta (TGF-β) and the related family of bone matrix proteins, act in bone remodelling and in skeletal development [Bonewald & Dallas, 1994; Sakou, 1998]. Fibroblast growth factors (FGFs) are important for physiological and skeleton repair bone remodelling [Kuznetsov et al., 1997; Inui et al., 1998].

3.2. Estrogens

Estrogens are a class of steroid hormones secreted mainly by the ovaries, and in smaller amounts also from placenta, adipose tissue, testicles and the adrenal glands. After menopause, their biosynthesis in the ovaries is minimal and circulating estrogens derive mainly from the peripheral aromatization of androgens. Aromatase is a key enzyme of estrogen biosynthesis as it catalyses the formation of estradiol from testosterone (Fig. 10). In nature, estrogens are found in various structurally related forms among which 17-β-estradiol (17β-E2) is the most predominant.

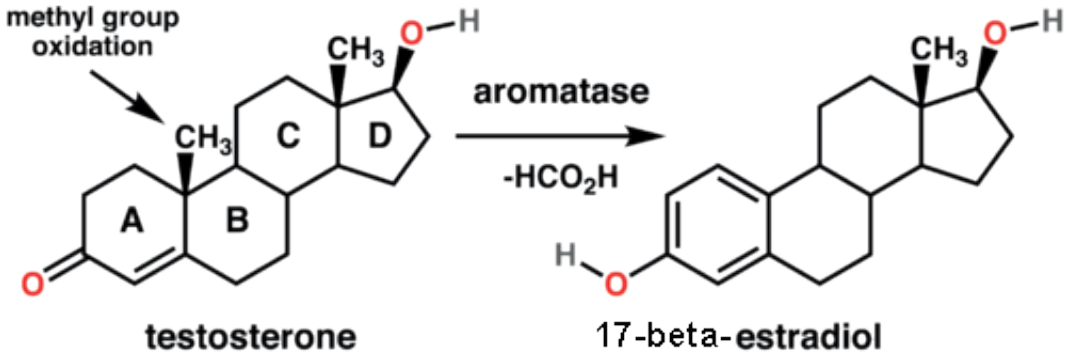


Figure 10. 17β-E2 synthesis from testosterone.

Their action is mediated by the specific estrogen receptor (ER), a dimeric protein that, after being activated by estrogen, moves into the nucleus where it binds to specific DNA sequences and controls gene expression. There are two different ER isoforms coded by different genes, defined as ERα and ERβ, capable of forming homodimers (ERαα, ERββ) and heterodimers (ERαβ). They are co-expressed in many cell types, such as bone and bone marrow cells. These receptors contain different domains including N-terminal domain, central domain for DNA, specific domain for estrogen and C-terminal domain. They do not only act as estrogen receptors but also have important functions in the absence of estrogens, such as regulating their production. The existence of another receptor, called ERRα, has been shown to be involved in the differentiation of osteoblasts. Estrogens may also bind to receptors located on the plasma membrane and thus generate a signal transduction by triggering the production of cyclic nucleotides, calcium flow and cytoplasmic kinase activation. The activation of these kinases, in turn, leads to the phosphorylation of proteins and transcription factors that possess regulatory effects on genes. However, gene regulation by estrogens can also occur through their direct binding to DNA, although more genes result to be regulated through the non-genotropic mode of action.

Estrogens exert potent influences on the size and shape of the skeleton during growth, and contribute to skeletal homeostasis during adulthood. Studies on osteoblast-like cell lines and on primary osteoblasts, as well as on estrogen-deficient rats and mice, suggested that estrogens might have an indirect effect on

both osteoclast formation and osteoblast-mediated bone resorption [Manolagas & Jilka, 1995; Khosla et al., 2012]. Estrogen deficiency increases the rate of bone remodelling, osteoclastogenesis, osteoblastogenesis, osteoclast and osteoblast numbers, as well as bone resorption and formation, thus resulting in resorption exceeding formation, and consequent bone fragility [Manolagas et al., 2002]. Conversely, estrogens restrain the rate of bone remodelling and maintain a balance between bone formation and bone resorption. The attenuation of the remodelling rate is also due to their effect on the formation of osteoblast and osteoclast progenitors, as well as to proapoptotic effect on osteoclasts and antiapoptotic effect on osteoblasts and osteocytes. Consistent with this statement, osteoblast apoptosis is increased in mice with deletion of ERα in mature osteoblasts and osteocytes. However, bone mass was not altered in this model, suggesting the lack of effect of ERα deletion in these cells.

Other studies have demonstrated that estrogens inhibit nuclear factor kappa-B (NF-κB) and reduce the production of osteoclastogenic cytokines by osteoblasts [Manolagas & Jilka, 1995]. Estrogens also regulate the RANK signalling pathway in osteoclastic cells by inducing apoptosis of osteoclasts [Kearns et al., 2008; Tang et al., 2009; Nakashima et al., 2011; Xiong et al., 2011]. This effect is related to the ability of estrogen to increase phosphorylation of extracellular signal-regulated kinases ERK1 and ERK2 and to suppress JNK activity [Marathe et al., 2011]. In osteoblasts cultured in the presence of an estrogen antagonist, estrogen induced the expression of its receptor ER, OPG and suppressed the expression of RANKL [Bord et al., 2003].

3.3. Pathogenesis and therapy of osteoporosis

Bone disease	Characteristics
Osteoporosis	Bone loss
Osteopetrosis	Dense bones
Osteogenesis imperfecta	Brittle bones
Paget's disease	Weak bone
Arthritis	Inflammatory joint disease
Rickets	Defective bone mineralization
Cancer	Malignant bone tumor
Osteomyelitis	Bacterial infection

Table 2. Metabolic bone diseases.

Rate of bone turnover, collagen synthesis, bone structure, bone geometry, bone density, are all parameters which influence skeleton mechanical support. Alteration of these parameters may cause diseases of bone remodelling or metabolic bone diseases, such as osteoporosis, osteopetrosis, osteogenesis imperfecta, Paget's disease, arthritis, rickets, cancer, osteomyelitis (Table 2). Causes of these diseases involve genetics, sex hormone deficiency, chronic presence of inflammatory and oxidative state, nutritional deficiencies, immobilization and pharmacological therapies (ie. glucocorticoid therapy) [Hardy, 2009].

The most common bone metabolic disease is osteoporosis (Fig. 11). This term literally means "porous bone" and it has been known to exist since the Egyptian mummies have been found with suspected kyphosis – the consequence of osteoporosis of the spine. It is characterized by progressive loss of bone density followed by alterations of bone microarchitecture. It is also known as "the silent thief" because the gradual loss of bone associated with this disease usually occurs over the years, and there are no noticeable symptoms until the bones become so fragile that a fracture occurs. It is caused by the imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. With advancing age, the balance between bone formation and bone resorption

progressively shifts towards resorption, leading to loss of bone mass and bone strength. This is consistent with the idea that aging leads to decrease in osteoblast number and increase in osteocyte apoptosis, which might increase the release of RANKL by healthy osteocytes in the vicinity, leading to increased bone porosity. Loss of estrogen during menopause leads to down-regulation of ERα expression in osteoclasts and osteoblasts, and therefore to a decrease in responsiveness of these cells. Increased oxidative stress has been revealed as the protagonist of this event. Age-related bone loss in females and males begins immediately after achieving peak bone mass, and this occurs long before and independently from the decline of sex hormones. However, at menopause this situation worsens and bone loss accelerates. On the other hand, men have a lower probability to suffer fragility fractures, possibly because they gain more bone mass during puberty and do not experience the abrupt estrogen loss as women do.

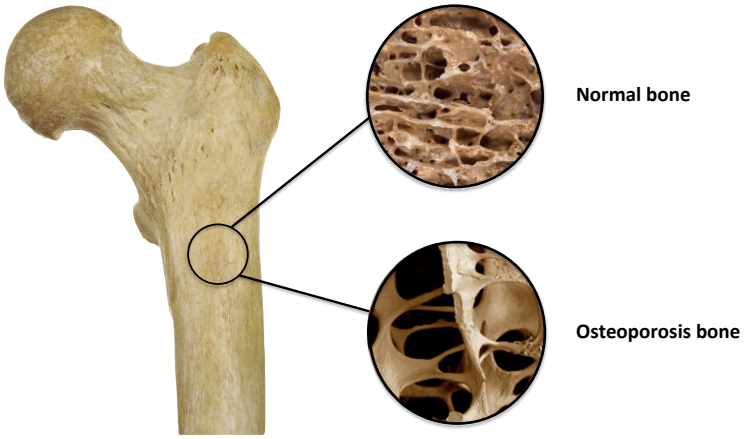


Figure 11. Normal and osteoporosis bone.

There are three types of osteoporosis:

- type I primary osteoporosis: occurs in post-menopausal women;
- type II primary osteoporosis: occurs in elderly individuals from 7th to 9th decade of life;
- secondary osteoporosis: is a secondary effect of drug therapy (ie. corticosteroids).

Week bones, related to less bone tissue mass, are more prone to fractures. Individuals suffering from fractures are at higher risk of death, not for the fracture itself, but because of the complications that may occur during hospitalization, such as immobilization, pneumonia, pulmonary thrombosis and embolism. Indeed, approximately 1 of 2 women and 1 of 5 men older than 50 years will eventually experience osteoporotic fractures. This is the reason why osteoporosis represents a major public health threat.

Currently, no treatment can completely cure osteoporosis (Table 3). However, early intervention can prevent osteoporosis in most people. For patients with osteoporosis therapy should be individualized and risks and benefits discussed between the clinician and patient. Patients at risk of osteoporosis and those who must take glucocorticoids, should undergo calcium and vitamin D intake as well as physical exercise.

Pharmacological therapy	
Hormone replacement therapy	Estrogen and progesterone
Selective estrogen receptor modulators	Raloxifene
Bisphosphonates	Alendronate, Risedronate, Ibandronate, Zoledronic acid
Human parathyroid hormone recombinant	Teriparatide
Calcitonin	
Monoclonal antibodies against RANKL	Denosumab
Anti-sclerostin antibody	Romosozumab
Strontium ranelate	
Recombinant human protein rhBMP-2	
Chitosan-nano-hydroxyapatite scaffolds	
Coral skeletons	
Microcrystalline hydroxyapatite supplement	
Osteoprogenitor stem cells	

Table 3. Pharmacological therapy for osteoporosis.

- Hormone replacement therapy with estrogen and progesterone was once considered the treatment of choice in post-menopausal women suffering from osteoporosis. The adverse outcomes associated with this therapy include breast cancer, myocardial infraction, stroke and venous thromboembolic events.
- Selective estrogen receptor modulators (SERM), such as Raloxifene, are synthetic molecules that act in bone as estrogen agonists and bind to its receptors, whereas in other tissues act as estrogen antagonists. Raloxifene may be the most useful in younger postmenopausal women without severe osteoporosis. SERM do not cause breast cancer.
- Bisphosphonates attach to hydroxyapatite binding sites on bone surface, especially on surfaces undergoing active resorption. When osteoclasts begin to resorb such bone, the bisphosphonates released during resorption impair the ability of osteoclasts to undergo activation changes. Alendronate is approved for treatment of osteoporosis in men, post-menopausal women and glucocorticoid-induced osteoporosis. Risedronate and Ibandronate are oral bisphosphonates. Zoledronic acid is the most potent bisphosphonate available given as once-yearly intravenous infusion. Complications of bisphosphonates include osteonecrosis of the jaw, atypical femur fractures, therefore, length of treatment with these medications should be considered.
- Human parathyroid hormone recombinant or Teriparatide, has the same physiologic action on bone and kidneys as PTH hormone. It acts as an anabolic agent for the treatment of osteoporosis in postmenopausal women and in men with hypogonadal osteoporosis who are at high risk of fracture and intolerant to other therapy. It cannot be given for a period longer than 2 years otherwise it leads to a neat bone loss due to increased osteoclastic and osteoblastic turnover.
- Calcitonin-salmon is indicated for the treatment of women who are more than 5 years post menopause and have low bone mass respect to healthy postmenopausal women, and of patients who refuse or cannot tolerate estrogens.
- Monoclonal antibody against RANKL or Denosumab, decreases bone resorption by inhibiting osteoclast activity. It is indicated for men and postmenopausal women who are at high risk of fractures or have multiple risk factors for fracture. It may be considered in certain patients with renal insufficiency, in men who receive androgen deprivation therapy for non-metastatic

prostate cancer, in patients with multiple myeloma or bone metastases, or in patients with autoimmune and inflammatory disorders.

- Anti-sclerostin antibody or Romosozumab increases bone formation and decreases bone resorption.
- Strontium ranelate is approved for the treatment of osteoporosis in some European countries but not in the United States.
- Recombinant human protein rhBMP-2, is an absorbable collagen sponge carrier, and if added as surgical implantation has been evaluated as a worthy candidate for periodontal regeneration.
- Chitosan-nano-hydroxyapatite scaffolds are used for bone regeneration.
- Coral skeletons can be transformed into hydroxyapatite under high temperatures. Their porous structure promotes bone ingrowth into prosthetic implants, while high temperature eliminates all organic residues, which may cause an immune response and rejection.
- Microcrystalline hydroxyapatite is used as supplement.
- Osteoprogenitor stem cells are a prospect approach in fracture repair and osteoporosis.

4. Oxidative stress and bone remodelling

Many studies performed on osteoblasts and osteoclasts have linked ROS and antioxidants to bone metabolism and bone remodelling [Mody et al., 2001; Bai et al., 2004; Lean et al., 2005; Jun et al., 2008]. In fact, intracellular oxidative state affects osteoblastic differentiation [Romagnoli et al., 2013] and osteoclasts physiologically generate ROS that contribute to bone degradation [Yang et al., 2001; Banfi et al., 2008]. However, ROS, and in particular H_2O_2, have a central role in bone loss caused by estrogen deficiency, and this has been related to increased osteocyte apoptosis [Kikuyama et al., 2002; Mann et al., 2007; Banfi et al., 2008; Jilka et al., 2013]. In fact, an increase in intracellular oxidative state occurs in ageing [Finkel & Holbrook, 2000; Nicks et al., 2012], in which the progressive loss of bone mass is also associated with osteocyte apoptosis [Henriksen et al., 2009; Jilka et al., 2013]. Similarly, chronic glucocorticoid treatment involving over-production of ROS is related to osteocyte apoptosis [Weinstein et al., 2000; Bjelakovic et al., 2007]. In fact, the antioxidant effect of estrogen is critical for osteocytes that are vulnerable to hypoxia, an event related to ROS increase [Rochefort et al., 2010]. Oxidative stress, due to abnormally high levels of oxidants and/or low levels of antioxidants, characterizes conditions that include postmenopausal osteoporosis, ageing, and inflammatory articular disorders [Finkel & Holbrook, 2000; Lean et al., 2005; Tilg et al., 2008].

ROS regulate intracellular signalling pathways by redox mechanisms (Fig. 12). In bone, H_2O_2 mediates a highly regulated oxidation of proteins involved in cell differentiation, thus modulating their activity. Increased ROS production causes phosphorylation of some of the proteins involved in Wnt/β-catenin signalling pathway leading to inhibition of Wnt-induced osteoblastic gene expression [Almeida et al., 2007]. As a result, ROS may cause increased osteoblast and osteocyte apoptosis by inhibiting OPG expression [Manolagas & Almeida, 2007]. In the presence of ROS, NF-κB is activated by RANKL binding to its receptor RANK, leading to osteoclast differentiation and activation. Moreover, NF-κB causes a decrease in osteoblast activity. Therefore ROS play an important role in osteoclast and osteoblast differentiation [Ha et al., 2004]. Consequently, the turnover of the

bone remodelling process increases and this is indicated by an increase in RANKL/OPG ratio, an index of the intensity of bone resorption [Lean et al., 2003, 2005]. The regulation of the RANKL/OPG ratio levels is responsible of the maintenance of balance between bone resorption and bone formation. Increased levels of this ratio are correlated to increased turnover of bone remodelling due to increased resorption rate without adequate and proper bone formation. This event has been related to the pathogenesis of various skeletal diseases, including various forms of osteoporosis and bone diseases secondary to inflammation [Bonewald, 2011; Mulcahy et al., 2011; Bellido, 2014].

Recently it has been seen that ROS production, and subsequent activation of mitogen-derived protein kinases (MAPKs), mediate osteoclast differentiation stimulated by RANKL. MAPKs have important role in cell proliferation, differentiation and apoptosis. These proteins are divided into various subgroups including the ERK1/2, p38 and JNK. ERK1/2 play essential role in the differentiation of mesenchymal cells in osteoblasts, and support the formation of osteoclasts. In fact, ERK1/2 activation induces RANKL expression. The inactivation of ERK1/2 significantly reduces the expression of RANKL, delaying the formation of osteoclasts. *In vitro* mechanistic studies have shown that the transduction of mechanical forces into intracellular signals induces the activation of ERKs, mediated by integrin, cytoskeletal proteins and kinases, and hence an increase in vitality of osteocytes. The ERK signalling pathway plays a key role in regulating FGF, a major signalling molecule in bone cells [Kousteni et al., 2003]. FGF regulates osteoclastogenesis and bone formation by activating ERK1/2 and a signalling pathway that involves the α-isoform of protein kinase C [Miraoui et al., 2009]. Both ERK1/2 and p38 MAPKs induce phosphorylation and activation of Runk2, a crucial transcription factor for differentiation of osteoblasts [Ge et al., 2012]. Moreover, *in vitro* studies have shown that p38 MAPK acts by regulating the expression of specific osteoblastic genes [Ortuno et al., 2010]. In mice, p38 MAPK was necessary for proper skeletal development. In fact, the deletion of p38 reduces differentiation of osteoblasts and bone mass [Greenblatt et al., 2010], which result increased by the hyperactivation of p38 MAPK [Whitehouse et al., 2010]. It is also likely that an interaction between p38 MAPK and Wnt signalling pathway is very important for coordinating the expression of osteoblastic genes since p38 MAPK activates

Wnt/β-catenine [Chen et al., 2010], and vice versa [Caverzasio & Manen, 2007]. Therefore, p38 is very important for bone formation and is a target for preventing the loss of bone mass occurring in osteoporosis.

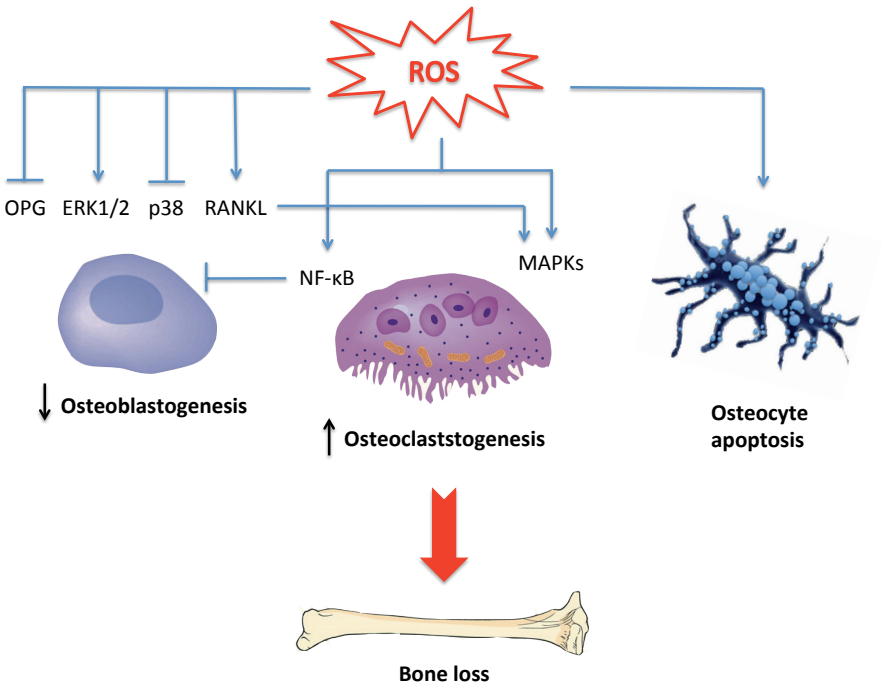


Figure 12. Main mechanisms involved in oxidative-stress induced bone loss.

Osteoclast and osteoblast function also depends on their interactions with extracellular matrix that through integrins modulate the differentiation and vitality of osteoblasts by activating the focal attachment kinases and ERK1/2 [Marie, 2009]. Recent studies have shown that osteocytes release FGF23 and a dentin matrix acidic phosphoprotein 1 (Dmp1), which regulate phosphate homeostasis. Dmp1 is abundantly expressed in osteocytes and is crucial for the differentiation of osteocytes themselves. Observations made *in vivo* and *in vitro* indicate that Dmp1 expression requires signalling pathways FGF and ERK [Makurami et al., 2004; Matsushita et al., 2009]. In the absence of ERK-1 and ERK-2, osteocytes do not develop characteristic dendritic prolongations. This shows that FGF and ERK play a key role in the differentiation and mineral osteocyte homeostasis [Kuyono et al., 2012]. The JNK signalling pathway is important for

many cellular processes and plays an essential role in differentiating osteoblasts [Stevens et al., 2010]. However, some molecules that induce osteoclastogenesis by increasing the expression of RANK, often express this function by activating JNK [Maeda et al., 2012]. In addition, JNK involvement has been demonstrated through the activation of caspase-3 in oxidative stress-induced apoptosis in osteocytes [Fontani et al., 2015].

4.1. Microdamage and osteocyte apoptosis

Osteocytes constitute 90% of the bone cell population and are embedded in the bone matrix. Their morphology is similar to neuronal cells, with a central body and dendritic extensions that serve as communicating appendages with other bone cells, blood capillaries and nerve endings. Osteocytes are considered the major regulators of bone remodelling and they play multifunctional roles that vary from mechanosensation and mechanotransduction [Tatsumi et al., 2007; Bonewald & Johnson, 2008], endocrine signalling through the secretion of FGF23 [Shimada et al., 2004; Bonewald et al., 2014], to orchestrating bone formation and bone resorption [Bonewald, 2011]. One of the most important events in regulation of the bone remodelling is the apoptosis of osteocytes (Box 1). Although the cause is unknown, the inhibition of this process causes the loss of osteocyte function. This evidence suggests that a normal rate of osteocyte death is crucial for maintaining bone health, and that artificial extension of osteocyte vitality is responsible for accumulation of cellular damage.

Box 1. Apoptosis features. Apoptosis or programmed cell death, takes its name from a Greek word *apoptoesis*, which indicates the fall of leaves in the autumn, and is a phenomenon that activates a series of biochemical modifications that cause elimination of cells. The understanding on the mechanism involved in this process transpired from the investigation of programmed cell death that occurs during the development of nematode *Caenorhabditis elegans*. Apoptosis occurs normally during development, ageing, as a homeostatic mechanism to maintain cell populations in tissues, as well as a defense mechanism when cells are damaged by disease or immune reactions. There are two main apoptotic pathways: the extrinsic or death receptor pathway and intrinsic or mitochondrial pathway. An additional pathway involves T cell –mediated cytotoxicity and granzyme B pathways. All the three apoptotic pathways converge on the same execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells macrophages. Macrophage digestion products are released into the extracellular space from where they are largely recovered from neighboring cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage.

Not only the vital osteocytes, but also the dying and dead ones can express markers and send signals that recruit and direct the function of osteoblasts and osteocytes, thus favouring normal bone turnover. When damage occurs within the

bone tissue, replacement is required. Microdamage is characterized by the lack of mechanical integrity in bone tissue. In fact, microdamage induces osteocyte apoptosis within the injured areas suggesting that osteocytes may be responsible for sending signals to osteoclast subpopulation surrounding those areas in order to initiate bone removal and repair [Kennedy et al., 2012]. Physiologically this is important in order to prevent accumulation and diffusion of the microdamage. Many studies show that osteocyte apoptosis due to microdamage is induced by bone fatigue [Verborgt et al., 2000], high impact intensity exercise [Warden et al., 2002], physical injury in bone and other tissues as well [Qui et al., 1997; Elmradi et al., 1999], estrogen deficiency [Jilka et al., 2013], increased age of an individual and infirmity [Schaffler et al., 1995], alteration of fluid flow with consequent disabled metabolic exchange and signal transmission throughout the canalicular system [Tami et al., 2002]. However, when osteocyte apoptosis becomes excessively high osteoclastogenesis prevails with consequent altered bone formation. The imbalanced osteoblast and osteoclast activity is correlated to an increased intracellular oxidative state [Noble et al., 2005; Rochefort et al., 2010; Fontani et al., 2015]. High levels of ROS are shown to decrease bone mineralization through the reduction of osteoblast activity and differentiation, as well as osteoblast and osteocyte apoptosis [Lee et al., 2006; Almeida et al., 2007; Henriksen et al., 2009; Bonewald, 2011, 2008; Jilka et al., 2013; Romagnoli et al., 2013]. This has been correlated mostly to estrogen deficiency occurring in ageing both in man and women [Finkel & Holbrook, 2000; Nicks et al., 2012], in postmenopausal women [Mann et al., 2007; Banfi et al., 2008; Jilka et al., 2013], in glucocorticoid treatment [Weinstein et al., 2000; Bjelakovic et al., 2007], in oxygen deprivation during immobilization [Dodd et al., 1999], as well as in pathological states associated with thiazolidinedione treatment [Mabilleau et al., 2010], in methotrexate treatment in cancer therapy [Shandala et al., 2011] and in alcoholism [Mauerl et al., 2011].

Studies of the bone marrow of aged mice revealed a reduction in antioxidant enzymes and an increase in phosphorylation of p53 and p66, markers of oxidative stress [Almeida et al., 2011]. In fact, under these conditions, the administration of NAC prevents accumulation of ROS and phosphorylation of p53 and p66.

Similar findings occur during marked estrogen decrease, which triggers oxidative stress related to menopause and subsequent bone loss [Manolagas et al.,

2013]. The decline in estrogens during menopause causes alteration of osteoblast mineralization, osteoclast resorption activity, as well as osteoblast and osteocyte apoptosis in both trabecular and cortical bone. It has been seen many times that estrogen treatment prevents osteocyte apoptosis due to microdamage and/or oxidative stress, however, molecular mechanisms and biochemical signals have not yet been clarified.

The accumulation of apoptotic osteocytes is also a glucocorticoid-induced feature whose apoptotic effect is associated with oxidative stress [Almeida et al., 2011; Jia et al., 2011], mediated by receptors and related to the activation of signalling pathways such as JNK [Plotkin et al., 2007]. In addition, glucocorticoids may contribute to the induction of osteocyte apoptosis by suppressing the survival factors such as IGF-1, IL-6 and MMPs [Canalis & Delany, 2002], and by inducing Secreted Frizzled Related Protein 1 (SFRP-1), antagonist of the Wnt signalling pathway [Wang et al., 2005]. Unlike the pro-osteoclastogenic signalling resulting from other causes of osteocyte death, glucocorticoid-induced osteocyte apoptosis may not be involved in increasing osteoclast number. This could be due to decrease of cytokine expression mediated by glucocorticoids. Glucocorticoid excess also causes adverse effects on the vascularity of bone because it causes a reduction in blood flow and water present in the skeletal vasculature and osteocyte canalicular system [Weinstein et al., 2010]. All these features account for the loss of bone strength that occurs before loss of bone mineral density (BMD) and the observed mismatch between fracture risk and BMD [Weinstein, 2011].

Mechanical loading also influences the vitality of osteocytes. The lack of mechanical stimulation [Noble et al., 2003; Lin et al., 2009], or an excessive load, may be responsible for an increase in osteocyte apoptosis in mice and rats [Verborgt et al., 2000; Canalis & Delany, 2002; Noble et al., 2003; Wang et al., 2005; Almeida et al., 2007, 2011; Plotkin et al., 2007; Jia et al., 2011]. In fact, mechanical loading has a profound influence on bone remodelling and the lack of load or excessive load causes an increase in bone turnover with prevalence of bone reabsorption. Lack of mechanical forces decreases survival signals induced by normal physical activity and, in particular, increases the expression of sclerostin, thus contributing to the increase of the death rate of osteocytes. It has been observed that close to microdamaged sites there is an increase in apoptosis of

osteocytes as bone turnover intends to replace and remove the injured tissue, and osteoclasts preferably gather in those regions. If damage runs faster than repair, microfissions are formed and can be propagated to form fractures. Microtraums and interruption of osteocyte canalicular network appear to play an important physiological role. High presence of pro-apoptotic molecules in osteocytes present in the vicinity of microtraums has been highlighted, and antiapoptotic molecule Bcl-2 is expressed 1-2 mm away from microtraums [Noble, 2000]. These findings suggest that the apoptotic area is limited to the microdamaged zone by activation of the protective antiapoptotic signals that avoid the further spread of cell death.

Signals transmitted, following apoptosis of the osteocytes, propagate through the canalicular network present on the surface of the bone tissue and are sent to progenitor cells. The nature of these signals is unknown, probably consists of fluid movements, biochemical signals or electric stimuli [Klein-Nulend et al., 1997; Noble, 2000], and are responsible for initiating bone remodelling cycle. In fact, osteocyte apoptosis regulates the recruitment of osteoclast precursors and stimulates osteoclastogenesis by direct or indirect mechanisms. The programmed osteocyte death results in increased RANKL secretion by osteocytes, stromal cells and osteoblasts. Such increase may also be due to the activation of the PTH receptor present on osteocytes. Osteocytes may also secrete OPG, which binds to RANKL and inhibits osteoclastogenesis. OPG secretion in osteocytes is regulated by Wnt/β-catenin. Studies on β-catenin deficiency in osteocytes revealed an osteoporotic pattern due to an increase in osteoclast number and bone absorption. It has also been shown that osteocytes may represent an additional source of M-CSF thus incrementing osteoclastogenesis. *In vivo* studies conducted on osteoclastic cells, have shown that the recruitment of these cells is also mediated by high mobility group 1 (HMGB1), a non-histone chromatin structural protein, released by the apoptotic osteocytes [Yang et al., 2008]. In addition to this, due to its pro-inflammatory action, HMGB1 may activate glycate receptors and Toll-like β3 receptors, thus inducing osteoclastogenesis [Zhou et al., 2008], may also act as chemotactic for osteoclasts, and increase RANKL/OPG ratio by stimulating RANKL expression and decreasing OPG levels [Kramer at al., 2010].

4.2. Antioxidants and oxidative stress in bone remodelling

Given the importance of oxidative stress in bone turnover, there is an increase in interest for their counterpart, the antioxidants, as an important strategy for mitigating or suppressing bone loss. Many studies *in vitro* and *in vivo* have been reported on the role of oxidative stress in osteoblast and osteoclast functions. From 2002, these studies have increased exponentially. They all suggest an important role of antioxidants in abrogating the effects of oxidative stress in bone and in contributing to osteoblast differentiation, mineralization and reduction of osteoclast activity. Antioxidants may either act directly, as ROS scavengers, or indirectly, as enzymatic defence systems [Franco et al., 2007; Jun et al., 2008; Romagnoli et al., 2013]. Maggio et al. have demonstrated that antioxidant defences were markedly decreased in osteoporotic females. Moreover, Sendur et al. have reported higher serum malondialdehyde (MDA) levels in the osteoporotic group than those in the control group. MDA is the end product of lipid peroxidation, one of the most damaging consequences of ROS activity, and a potential biomarker for oxidative stress. The loss of antioxidants leads to increased bone loss through the activation of a TNFα-dependent signalling pathway [Lean et al., 2005], and the administration of antioxidants such as vitamin C and E, NAC and lipoic acid (LA), has beneficial effects in individuals with osteoporosis [Hall & Greendale, 1998; Morton et al., 2001; Sanders et al., 2007; Mainini et al., 2012]. In ovariectomized mice, NAC, vitamin C and LA abrogate the ovariectomy-induced bone loss, while L-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of glutathione synthesis, causes substantial bone loss [Lean et al., 2003; Polat et al., 2013]. Furthermore, NAC supplementation with diet has been shown to mitigate bone loss, down-regulation of Wnt signalling and decrease in bone-formation markers in the ethanol-induced oxidative stress mouse model [Chen et al., 2010]. A recent Chinese study in over 3000 adults, has highlighted that vitamin E is able to ameliorate bone mineral density in middle-aged and elderly women but not in men [Shi et al., 2016]. Moreover, it promotes healing of osteoporotic fracture in ovariectomized rats by inducing bone regeneration [Shuid et al., 2011].

As regards antioxidant direct scavenging activity, some date have been obtained using NAC in cell cultures, where it controls oxidative stress in many cells including osteoblasts, inhibits their apoptosis, and stimulates osteoblastic differentiation of mouse calvarial cells by GSH involvement, being NAC its precursor [Jun et al., 2008; Ueno et al., 2011; Romagnoli et al., 2013]. Similarly, NAC prevents osteoclast formation, NF-κB activation and TNF-α expression involved in osteoclast activation [Lean et al., 2003]. Moreover, GSH and NAC increase alkaline phosphatase (ALP) activity and other osteogenic markers, such as Runt-related transcription factor 2 (RUNX-2) and osteocalcin, during the differentiation of these cells into mature osteoblasts [Romagnoli et al., 2013]. They also decrease RANKL/OPG levels in SaOS-2 cells, and increase calcium levels and mineralization process [Romagnoli et al., 2013]. Therefore, these antioxidants are able to restore mineralization process due to oxidative stress and have anti-osteoclastogenic action. LA also suppresses osteoclastogenesis in hBMSC cells by direct inhibition of RANKL-RANK mediated signals [Koh et al., 2005]. Fontani et al. 2015 have recently demonstrated the ability of GSH, NAC and LA to prevent osteocyte apoptosis and the increase of sclerostin and RANKL/OPG ratio levels induced by oxidative stress. Following are summarized the effects of antioxidants against the oxidative stress at the molecular and cellular level (Fig.14). At the molecular level, antioxidants prevent RANKL and sclerostin increase and OPG decrease, thus inhibiting the enhancement of the RANKL/OPG ratio in osteoblasts and osteocytes. Moreover, in osteoclasts they inhibit the increase of bone acid phosphatase and protease activity, which both degrade the bone matrix, whereas in osteoblasts they induce alkaline phosphatase and matrix protein synthesis. At the cellular level, they counteract the excessive apoptosis of osteoblasts and osteocytes and reduce the differentiation and activity of osteoclasts.

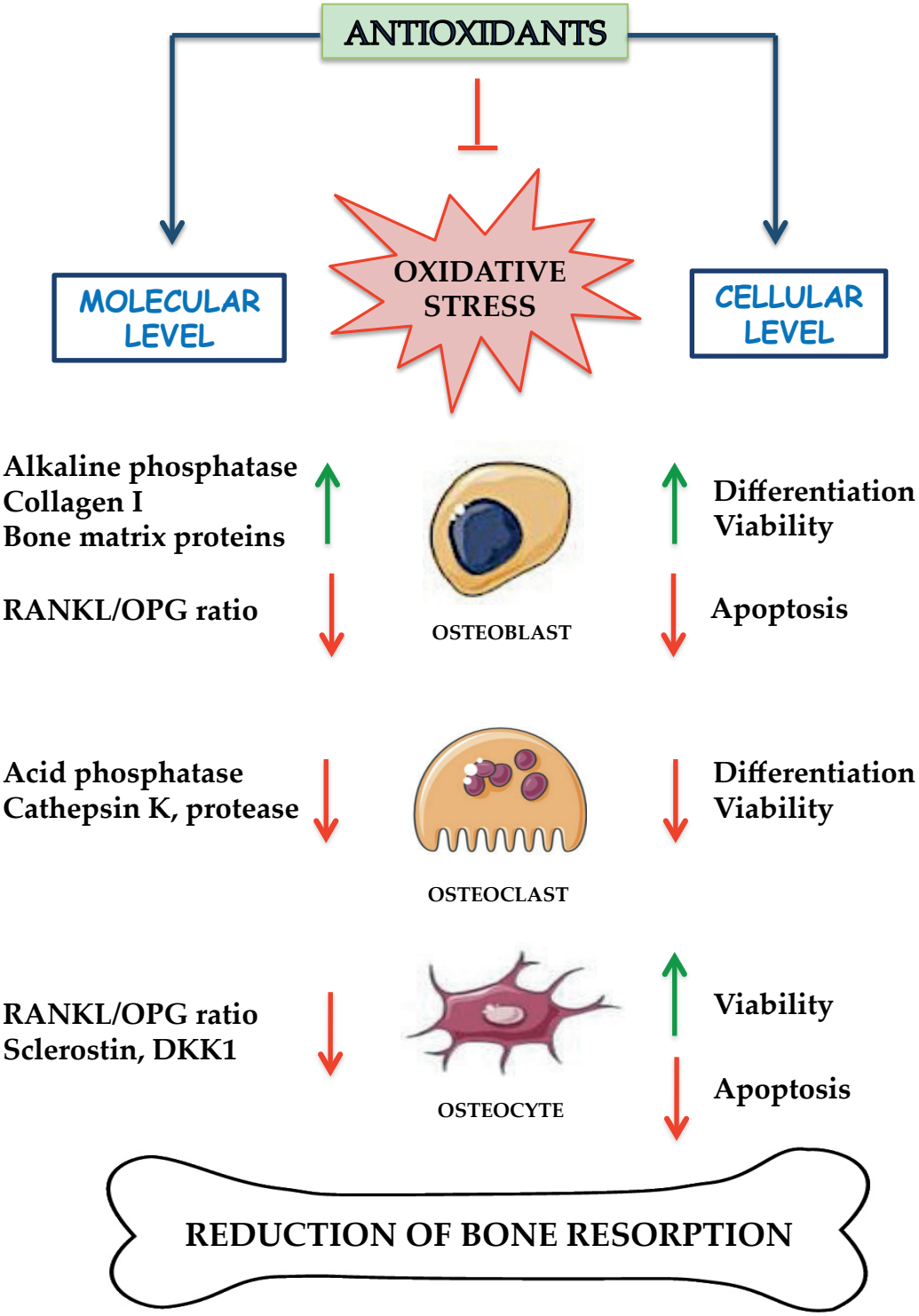


Figure 14. Summary of the effects of antioxidants on bone remodelling process.

Considering the possible adverse side effects associated with certain medication such as bisphosphonates and estrogen therapy used by postmenopausal women, there is a rising demand for use of natural antioxidants instead of medical therapy in prevention and improvement of this progressively debilitating condition. This is of particular benefit for poly-medication consumption as natural compounds may be used as adjuvant therapy or supplementation. Therefore, nutritional approaches to antioxidant therapy have recently been tested in cell cultures, animals and selected group of patients suffering from osteoporosis or inflammatory bone diseases, ultimately demonstrating their usefulness in bone development as well as in prevention and treatment of bone loss diseases.

The most abundant phytochemical antioxidants deriving from diet are polyphenols, the most prominent of which being anthocyanins - the widespread constituents of fruits (especially blueberries), vegetables, cereals, dry legumes, chocolate, tea, coffee and wine. For example, polyphenol total dietary intake can range up to 1 g/die, which is considerably higher than that of other phytochemical antioxidants [Velioglu et al., 1998]. Anthocyanin dietary intake was estimated as 12 mg/die in the US population [Wu et al., 2006]. Higher consumption of fruits and vegetables has been correlated to a reduction in the risk of development of osteoporosis. There have been several results suggesting that the combination of polyphenol compounds found naturally in fruits and vegetables may reduce the risk of osteoporosis and increase BMD. In particular, blueberry polyphenols due to their antioxidant power improve osteoporosis condition in a rat model of ovariectomy-induced osteoporosis, which is the most common cause of age-related bone loss [Li et al., 2014]. Various studies have reported that blueberry intake can increase antioxidant status, reduce the level of inflammatory cytokines (IL-6, IL-15) and decrease MDA levels. In the presence of oxidative stress blueberry polyphenols activate the Wnt signalling pathway through p38 phosphorylation, thus leading to the inhibition of osteoclastogenesis [Weaver et al., 2012]. They also suppress bone resorption through RANKL down-regulation in bone marrow stromal cells [Zhang et al., 2013]. Moreover, at the transcription level, blueberry polyphenols decrease NF-κB expression increased during oxidative stress and counteract the effects of osteoclasts [Trzeciakiewicz et al., 2009].

Another popular polyphenol of stilbene structure is RE which antioxidant activity was studied in various tissues. Regarding the musculoskeletal system, it has been shown that RE was able to decrease oxidative stress by increasing catalase activity as well as SOD content and activity [Jackson et al., 2010]. Moreover, nutritional concentrations of RE are able to decrease H_2O_2 levels in MCF-7 cells by inducing the expression of antioxidant genes, such as of catalase and MnSOD, through a mechanism that involves phosphatase and tensin homolog (PTEN), a well-known tumor suppressor that antagonises the activity of phosphatidylinositol 3-kinase type I (PI3K), thus leading to decrease in protein kinase B (also known as Akt) phosphorylation [Inglés et al., 2014]. Resveratrol increases bone mineral density and bone alkaline phosphatase in osteoporotic obese man [Ornstrup et al., 2014], and represents an effective therapeutic agent in preventing bone loss in ovariectomized and old rats [Tou, 2015].

Various studies show that green tea drinking/ingesting may mitigate age-related bone loss in elderly men and women [Shen et al., 2009] as well as chronic inflammation-induced bone loss [Shen et al., 2010]. GT components inhibit formation and differentiation of osteoclasts, block RANKL signalling essential for osteoclastogenesis and induce osteoclast apoptosis [Nakagawa et al., 2002; Hafeez et al., 2006; Lin et al., 2009; Oka et al., 2012]. Moreover, GT stimulates osteoblastogenesis and bone mineralization [Chen et al., 2005; Vali et al., 2007]. These effects are related to the ability of GT polyphenols to capture and detoxify ROS. Recently, it has been demonstrated that GT polyphenols improve cellular antioxidant enzyme GPX in both 15-month-old sham and ovariectomized rats [Shen et al., 2008, 2009a]. In a study of Itoh et al., the most abundant GT polyphenol, (-)-epigallocatechin gallate, decreased the formation of abnormal calcium stone deposit induced by oxidative stress in rats.

Flavonoid-rich *Hypericum perforatum* extract exhibits a strong antioxidant activity. It prevented the peroxidation of lipid membranes in liposome and linoleic acid, and has demonstrated radical scavenging activity [Zou et al., 2004]. HP also has beneficial effects on osteoblast proliferation and estrogen deficiency-related bone loss in postmenopausal women [You et al., 2015]. Peculiar is the effect of HP on stress associated to mental strain and corporal exhaustion such as during cold swimming connected with bone mass loss [Seferos et al., 2016].

5. Oxidative stress in intestinal inflammation

5.1. Intestinal inflammation: interplay between immune and non-immune cells

Even under normal conditions the intestinal mucosa disposes a state of "physiological inflammation", which is maintained by the presence of leukocytes in the intra-epithelial and sub-epithelial compartments that trigger an immune response to diet and bacteria present in the intestinal lumen [Fiocchi, 1994]. Therefore, during the state of "pathological inflammation", the cells of mucosal immune system are responsible for such immune response. Two models have been proposed to explain this behaviour. The first model is unidirectional, by which activated immune cells, represented mainly by neutrophils, macrophages, and cytotoxic T cells, attack and destroy neighbouring cells. They can do it either directly through physical contact or indirectly by releasing soluble factors such as ROS and RNS, cytotoxic proteins, lithic enzymes or cytokines [Grisham et al., 1992; Fiocchi et al., 1994]. In this model, non-immune mucosal cells do not participate but only "witness" the destruction process operated by the immune cells (Fig. 15).

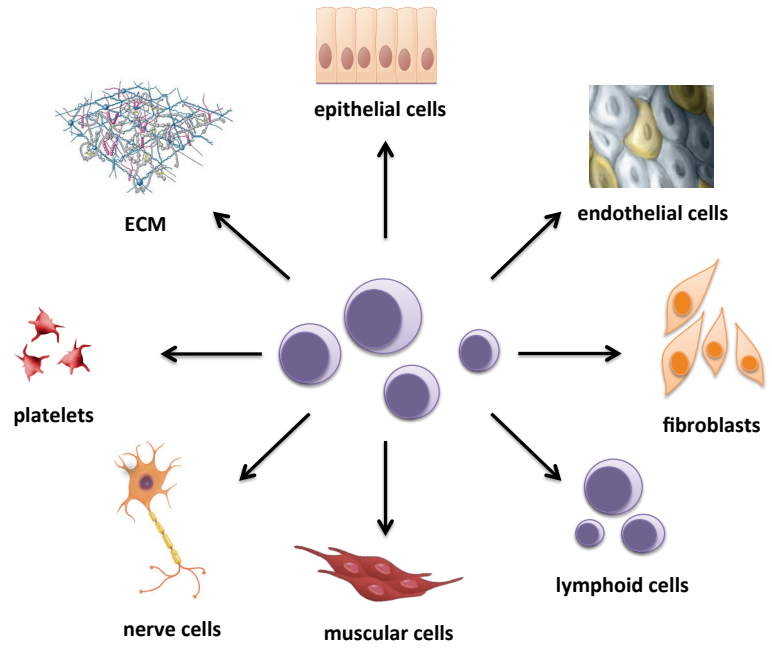


Figure 15. Unidirectional model of interaction between cells involved in intestinal inflammation: activated immune cells are main actors, while non-immune cells are simply the spectators.

This model, however, gave way to a multidirectional model because of growing evidence on complex network of specialized cell types and the level of cell-cell communication that underlie immunological reactions (Fig. 16). In according to this model, cellular and non-cellular elements in the intestinal mucosa, such as epithelial, endothelial, mesenchymal, nervous and vascular cells, platelets, fibroblasts, myofibroblasts and the extracellular matrix, do not behave as simple witnesses but are actively involved in the intestinal immune and inflammatory response under both physiological and pathological conditions [Danese, 2008]. This model results more appropriate for investigating the intestinal inflammation mechanisms, since non-immune cells are implicated in symptoms and structural changes such as pain, dismotility, fibrosis, stricture, fistula formation, obstruction and neoplastic transformation.

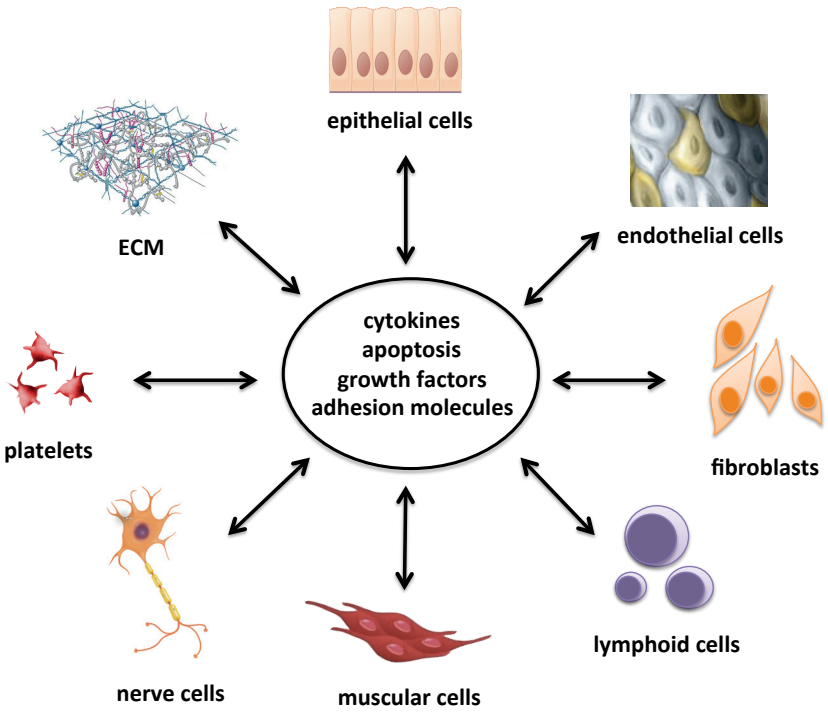


Figure 16. Multidirectional model of interaction between cells involved in intestinal inflammation: reciprocal interactions between immune and non-immune cells.

Epithelial cells are not only a barrier, but they are involved in the recognition of pathogenic bacteria and tolerance towards the commensal bacteria. Intestinal epithelial cells are also antigen-presenting cells and express the major histocompatibility class (MHC) I and II molecules as well as non-classical MHC molecules, which expression increments in response to pro-inflammatory stimuli and can potentially trigger CD4+ T cells [Buning et al., 2006]. Epithelial and immune cell interaction is bidirectional and involves IκB kinase and the expression of indoleamine 2,3-dioxygenase (IDO) recently been demonstrated to enhance apoptosis of T cells and suppress their proliferation [Jasperson et al., 2008].

Microvascular endothelium is responsible of the regulation of type and number of leukocytes migrating from the vessels to the interstitial space, through the secretion of chemokines and the expression of cell adhesion molecules. Altered function of microvessels affects the function of endothelial cells, changes the expression of genes whose products regulate coagulation, extracellular matrix (ECM) remodelling, angiogenesis and permeability to leukocytes. Recently, lymphatic vasculature also plays a role in immunity; it regulates the homing and migration of lymphocytes, by producing specific chemokines, and expresses vascular endothelial growth factor (VEGF) specific for lymphatic vessel growth [Wu et al., 2005].

Platelets are important inflammatory mediators, and endothelial cells stimulated with pro-inflammatory cytokines can activate them. Their activation abnormalities are present in chronic intestinal inflammation [Danese et al., 2004]. It has been seen that *in vitro*, platelet activation up-regulates adhesion molecules involved in leukocyte adhesion, chemokines and p38-mediated T-cell recruitment [Danese et al., 2003].

Nervous system innervates the intestinal mucosa and the submucosa and ensures the transfer of signals between the nervous system and the immune cells, and vice versa. The stimulation of sensory nerve fibers by cytokines produced by leukocytes triggers sympathetic and parasympathetic nervous system [Del Rey et al., 2003; Besedovsky & Rey, 2007] with consequent production of adrenaline and norepinephrine. These influence vitality of intestinal lymphocytes [Marra et al., 2005] and inhibit immune functions of macrophages and lymphocytes [Calcagni & Elenkov, 2006; Elenkov, 2007]. The substance P (SP), a key factor in immune

regulation released from both nerve fibers and immune cells [Riegler et al., 1999; Brun et al., 2005], binding to neurokinin-1 (NK-1) receptor activates NF-κB in target cells leading to increased expression of pro-inflammatory genes. These communicational processes based on the work of pro-inflammatory mediators, have also been associated with intestinal inflammation.

Intestinal fibroblasts and myofibroblasts are situated underneath a layer of antigen-sampling epithelial cells, and surrounded by antigen-responsive immune cells. They can initiate inflammation by producing cytokines and cell adhesion molecules that enhance interactions with immune or non-immune cells. Fibroblasts produce also ECM components that have protective and anti-inflammatory role leading to reconstruction of epithelial cells [Mahida et al., 1997], and are important for the function and longevity of T cells [Sturm et al., 2004, 2004a]. Fragments of degraded ECM can also have pro-inflammatory functions [Vaday et al., 2000].

5.2. Myofibroblasts and chronic intestinal inflammation

Intestinal myofibroblasts are one of the several mesenchymal cells in the intestinal *lamina propria*. The term "mesenchyme" originates from the Greek *mesos* (= intermediate) and *énchyma* (= infusion), and is used to define a tissue that fills the space between two functionally different tissues, in this case, epithelium and smooth muscle. The mesenchyme of the intestinal *lamina propria* has a layered structure and is characterized by the presence of ECM and cellular components, such as intestinal fibroblasts, myofibroblasts and pericytes, located in each layer and along the crypt/villus axis [Furuya S & Furuya K, 2007; Powell et al., 2011]. Mesenchymal cells appear during normal wound healing after inflammatory processes, although their persistence is associated with excessive collagen deposition, mesenchymal cell hyperplasia and fibrosis, all events associated with IBD.

There are two different myofibroblast populations inside *lamina propria*: interstitial cells of Cajal (ICC) and intestinal sub-epithelial myofibroblasts (ISEMFs). ICCs are pacemaker cells, located in the intramuscular space between

the submucosa and the *muscularis propria*, which regulate the motility of smooth gastrointestinal muscle cells and facilitate the propagation of electrical signals and neurotransmission [Sanders et al., 2002]. ISEMFs, also called pericryptic fibroblasts, are located at the base of intestinal crypts in their own lamina and form a three-dimensional network in connection with epithelial cells [Powell et al., 1999]. ISEMFs cells are of great interest in regulating the proliferation and differentiation of epithelial cells and are involved in mucous membrane protection, wound healing, transport of water and electrolytes, as well as in ECM metabolism that influence the growth of the basal membrane [Powell et al., 1999, 1999a; Andoh et al., 2005a, 2005b].

Myofibroblasts represent fibroblasts with smooth muscle cell characteristics (Fig. 17). Therefore, they are able to contract and thus play a crucial role in remodelling and regeneration of the damaged tissue. During wound healing, there is a gradual transition from fibroblasts to contractile myofibroblasts in which fibroblasts firstly evolve in proto-myofibroblasts, which express stress fibers containing β- and γ-actin, and then completely differentiate in myofibroblasts, which express α-smooth muscle actin (α-SMA) considered a typical myofibroblast differentiation factor [Desmouliere et al., 2005]. However, fibroblast marker CD90 remains present in myofibroblasts, while smooth muscle cell marker, desmin, is absent [Pinchuk et al., 2010]. Moreover, myofibroblasts are located aligned below epithelial cells, connected to each other and to the surrounding matrix by fibronexus [Eyden, 2008; Mifflin et al., 2011]. This is an adhesion structure containing fibronectin filaments that run parallel to myofibroblasts and then cross into their cytoplasm. These adhesions make them a powerful contractile network important for reducing the size of the wound.

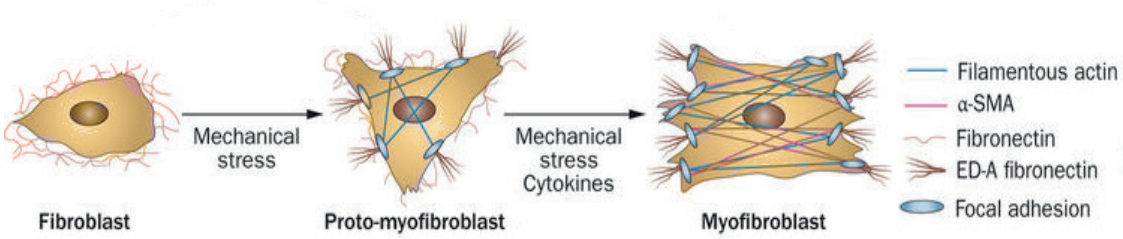


Figure 17. Transition from fibroblasts to myofibroblasts observed during wound healing.

Intestinal epithelium is highly susceptible to bacterial, physical and chemical attack that can lead to loss of its integrity. Regeneration of the epithelial barrier is therefore essential for the maintenance of intestinal homeostasis and for the prevention of an uncontrolled inflammatory response. In the process of repair, epithelial cells and myofibroblasts migrate into compromised area and reorganize ECM by exerting traction forces on it in order to promote wound healing [Tomasek et al., 2002]. In addition to this, myofibroblasts deposit collagen and express matrix-metalloproteinases (MMPs) that promote further remodelling of the ECM. In fact, their role in intestinal inflammatory processes has been mainly studied in relation to ECM remodelling process and MMP expression. Mediators that promote proliferation and ECM production by myofibroblasts are various and include growth factors, cytokines and endothelin (Fig. 18) [Wynn, 2008, 2007; Rieder & Fiocchi, 2009; Speca et al., 2012]. Myofibroblasts can also be activated by epithelial cells, by products derived from damaged cells, DAMs (damage-associated molecular patterns), and also by molecules produced by pathogenic microorganisms, pathogen-associated molecular patterns (PAMPs) [Rieder & Fiocchi, 2009]. Some *in vitro* studies have shown that stimulation of myofibroblasts with TNFα, cytokine with important role in the pathophysiology of IBD, induces the expression of MMP1, MMP3, tissue inhibitor of metalloproteinases 1 (TIMP-1) and secretion of type I and IV collagen [Okuno et al., 2002; Bamba et al., 2003]. It has also been shown that myofibroblasts isolated from the inflamed bowel of patients with CD present oxidative stress and overexpress MMPs, such as MMP2 [Romagnoli et al., 2013a] and MMP3 [Fontani et al., 2014], while myofibroblasts isolated from fibrotic intestine areas overexpress TIMP1 [McKaig et al., 2003]. Treatment with anti-TNFα antibodies increases the expression of TIMP-1 in myofibroblasts isolated from inflammatory intestinal tissue of patients with CD, and increases their migration [Di Sabatino et al., 2007].

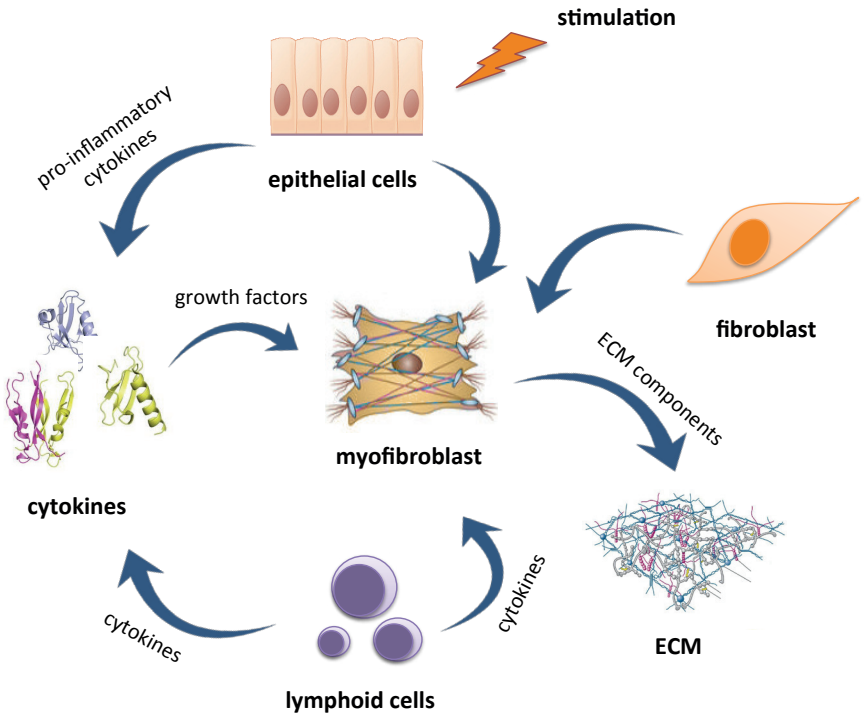


Figure 18. Mediators involved in the differentiation and proliferation of myofibroblasts and the production of extracellular matrix components.

Some *in vivo* studies show that intestinal myofibroblasts play a critical role in maintaining homeostasis in the damaged tissue by activating the Tpl2-COX-2-PGE2 pathway, which stimulates the proliferation of epithelial cells in the presence of an inflammatory state [Roulis et al., 2014]. In particular, inflammatory stimuli in myofibroblasts activate tumor progression locus-2 (Tpl2) kinase that induces the activation of cyclooxygenase-2 (COX-2) and the secretion of prostaglandin PGE2, involved in the proliferation of epithelial cells in the damaged tissue.

Very often, acute intestinal inflammation is followed by a physiological healing of the damaged tissue by regeneration process of the intestinal tissue [Rieder et al., 2012]. If this does not occur, chronic inflammation develops and continuous alterations of wound healing events can lead to fibrosis, characterized by an imbalance between ECM production and its degradation [Rieder et al., 2012]. In particular, intestinal fibrosis is a consequence of abnormal production of ECM by activated myofibroblasts [Burke et al., 2007; Rieder & Fiocchi, 2009, 2008; Speca et al., 2012].

5.3. Oxidative stress in chronic intestinal inflammation

Among the immune-regulatory factors, oxidative stress has been proposed as the most important event in the physiopathology of many intestinal inflammatory diseases including Inflammatory Bowel Disease (IBD) (Box 2). Substantial evidence demonstrate that in IBD an imbalance between ROS production and antioxidant defence system is present, causing damages to mucosa of the gastro-intestinal (GI) tract and bacteria infection, thus leading to an exacerbated anti-inflammatory response [Goyette et al., 2007].

Box 2. IBD features. Inflammatory bowel diseases deal with colon and small intestine incurable chronic inflammatory conditions, which include principally Ulcerative colitis (UC) and Crohn's disease (CD). CD is characterized by a transmural inflammation that may occur in a discontinuous pattern in any region of gastrointestinal (GI) tract involving ileum and colon. UC is restricted to mucosa and affects solely colon and rectum in a continuous pattern [Corridoni et al., 2014]. Specific features of CD are intestinal granuloma, stenosis and fistulas, not found in UC [Abraham & Cho, 2009]. Moreover, patients with CD and UC have an increased risk of developing malignancies such as colon cancer and small bowel carcinoma, the latter reserved only for patients with CD. Yet is a largely hidden disease, mostly affecting young people from 15 to 30 years old, many of which are undiagnosed and suffer in silence. The exact aetiology of IBD is not well known. There are several factors involved in development of IBD, including genetic variations, immune system alterations, intestinal microflora and bacterial infection [Abraham & Cho, 2009; Boirivant & Cossu, 2012].

The immune mechanism underlying IBD regards disrupted balance between T helper (Th) and T regulatory (Treg) cells due to dysfunction of Treg cells and consequent overproduction of pro-inflammatory cytokines by Th cells. The activation of immune cells leads to the production of ROS and RNS, and in particular of $O_2^{-\cdot}$ and $NO\cdot$, which in excessive amount can cause alterations of tight junctions and epithelial permeability. These species may also react together to form peroxynitrite, an extremely reactive specie found to be implicated in the pathogenesis of IBD [Pacher et al., 2007]. Studies on colonic biopsies of IBD patients showed that oxidative stress may be correlated to the degree of disease, and that the main sites for ROS production are phagocytic leucocytes after they infiltrate in the intestine mucosa causing inflammation [Dodorico, 2001]. Bacteria can penetrate in the damaged epithelial barrier and activate the expression of receptors that play a key role in the regulation of innate immune response, such as Toll-like and nucleotide-binding oligomerization domain (NOD)-like receptors, as well as cytokine receptors that can co-stimulate immune cells during inflammation [Senhai et al., 2015] and activate downstream NF-κB signalling by ROS generation [Jeong et al., 2009]. NF-κB can increase the transcription of five types of genes encoding for pro-inflammatory cytokines,

metalloproteinases (enzymes that digest mucosal cells), COX-2, NOS and NOX enzymes, as well as genes involved in epithelial cell apoptosis and in intestinal barrier permeability [Andersen et al., 2005; Qiu et al., 2011]. In particular NOX-1, are constitutively and highly expressed in colonic tissue, and ROS generated by them are potentially involved in the pathogenesis of IBD. The activation of NOX1 is associated to early disease change, and may happen prior to pathological changes.

The blood microvessels surrounding damaged epithelial cells can recruit inflammatory cytokines, leading to further tissue damage such as loss of epithelial cells, crypt cell hyperplasia, reduced mucus production and ulcerations. During inflammation, intestinal microbiota also represent an additional source of ROS production, contributing to further tissue injury [Rezaie et al., 2007].

Studies performed in CD patients demonstrated an association between oxidative stress and expression of fibrogenic cytokine, such as transforming growth factor beta (TGFβ) [Chiarpotto et al., 1997], growth factors, such as TGF-1 [Rezaie et al., 2006], epidermal growth factor (EGF) [Jahanashahi et al., 2004], platelet-stored and blood-circulating platelet-derived growth factor (PDGF) [Krzystek-Korpacka et al., 2009]. Moreover, the accumulation of mitochondrial DNA mutation due to mitochondrial ROS, contributes to increased susceptibility of colorectal cancer of patients with IBD [Dashdorj et al., 2013].

Chronic intestinal inflammation is characterized also by lipid peroxidation due to GSH depletion [Koch et al., 2000], altered lipid profiles, lipoprotein composition, oxidant and antioxidant levels [Levy et al., 2000]. In fact, decreased GSH and increased GPX levels in the plasma and intestinal mucosa of IBD patients have been correlated to the severity of intestinal inflammation [Iantomasi et al., 1994; Tuzun et al., 2002].

Smoking, as a lifestyle factor, can interact with oxidative stress in IBD development given its influence on ROS generation pathways and high concentration of ROS in gas and tar of cigarette smoke. Metal ions present in cigarette smoke can catalyse transformation of H_2O_2 in highly reactive $OH\cdot$, considering that smoking dramatically decreases SOD levels in the colon [Guo et al., 2001]. In the ‘90s, Lih-Brody et al. showed for the first time an increment of ROS, protein carbonyl contents, DNA oxidation products and iron, as well as a decrease of Cu^{2+} and Cu/Zn-SOD activity in CD biopsies.

5.4. TNFα and chronic intestinal inflammation

TNFα is a protein belonging to the family of cytokines, small proteins, with molecular weight between 6 and 60 kDa, implicated in cell interaction and communication pathways (Box 3). There are, in fact, several studies that associate the alteration of inflammatory cytokine production with pathological processes, such as rheumatoid arthritis, chronic intestinal inflammation, multiple sclerosis and lupus erythematosus [Ebert et al., 1984; Mitsuyama et al., 1991].

Box 3. Cytokine functions. Cytokines may have autocrine action by acting on the same cells that secrete them, paracrine action by acting on nearby cells, or endocrine action by acting on distant cells. Cytokines are secreted by different cell types and in particular by immune cells, such as Th cells and macrophages. In particular, inflammatory cytokines, such as IL-1b, IL-6, IL-8, IL-12 and TNFα, are mainly produced by activated macrophages and are able to exert many physio-pathological activities. A common feature to cytokines is pleiotropy, which is the ability to act on the same cell type with different functions, but also on completely different cells that have no common origin, function nor localization. This feature causes a wide range of cellular responses including proliferation, cell differentiation, angiogenesis and high inflammatory response.

TNFα is initially produced as a type II transgenic protein constituted by 212 aa, organized as a stable homotrimer and having a molecular weight of 32 kDa. The membrane-integrated form releases the 17 kDa soluble homotrimeric cytokine, thanks to the proteolytic cleavage operated by the metalloproteinase TNF-converting enzyme TACE also known as ADAM-17 [Kriegler et al., 1988]. TNF superfamily members express their activity by acting on membrane receptors such as the TNFR receptor family and in particular TNF-R1 and TNF-R2 (Fig. 19) [Locksley et al., 2001]. The two receptors are highly homologous in extracellular portion containing TNFα binding sites characterized by cysteine residues, but significantly different in intracellular portions. TNF-R1 is constitutive of many tissues while TNF-R2 is found in the immune system and its expression is largely regulated by it. Following TNFα extracellular binding, each receptor recalls within its own intracellular domain the cytosolic protein complex containing the TNF receptor associated death domain (TRADD), serine/threonine receptor interacting with protein kinase 1 (RIP) and ubiquitin kinase "TNFR-associated factor 2" (TRAF2). TRADD can also interact with the "Fas associated death domain protein" (FADD) and caspase-8, leading to the activation of caspase-3 and apoptosis. TRAF2, RIP and kB inhibitor (IKK) activate NF-kB, which mediates transcriptional control of genes involved in tumor cell proliferation, expansion and survival.

Furthermore, TNFα, by recruiting TRAF2, activates the JNK pathway, found enhanced in sites of active inflammation compared to normal intestine in patients with IBD [Mistsuyama et al., 2006].

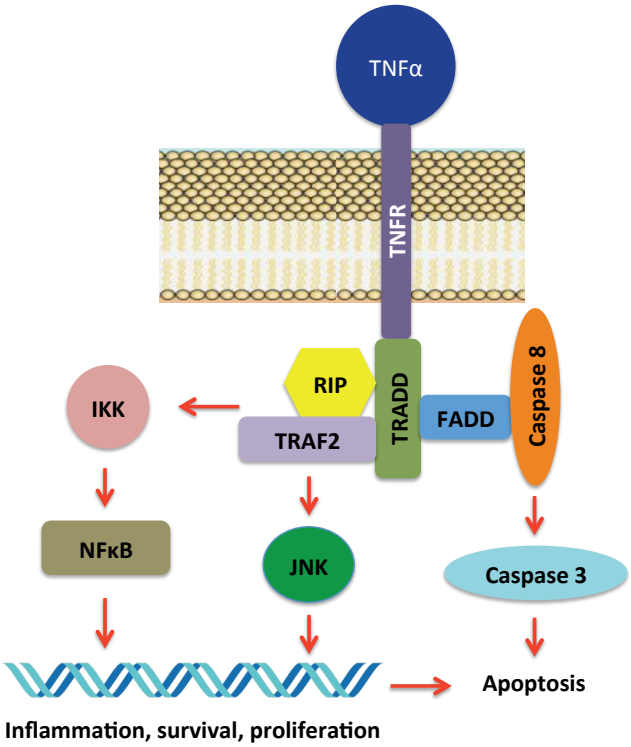


Figure 19. TNFα signalling.

In inflammatory processes, TNFα increases migration of leukocytes in inflammatory sites by inducing the expression of chemokines and adhesion molecules on endothelial cells, such as Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular cell adhesion protein 1 (VCAM-1). In addition, TNFα is involved in the overexpression of COX-2, causing the maximum release of prostaglandin PGI2 that, for its peripheral vessel-dilatation action, causes classic symptoms of inflammation, such as redness, swelling, heat and vascular permeability [Mark et al., 2001]. TNFα biological effects depend of the amount and rate of the release following proper stimulus; high release causes shock, tissue damage, intestinal necrosis, adrenal bleeding and fever, while prolonged release of low TNFα levels

involves a debilitating syndrome characterized by depression, anorexia, catabolism and insulin resistance.

Genetic studies suggest that TNFα plays a crucial role in the pathogenesis of IBD being abundantly expressed in the mucosa and contributing to intestinal inflammation through the alteration of the epithelial barrier and apoptosis of the epithelial cells with consequent damage to the intestinal tissue [Bosani et al., 2009]. Recent studies have shown that membrane-bound TNF, rather than soluble TNF, has a major contribution to intestinal inflammation. In particular, neutralization of membrane-bound TNF induces Th cell apoptosis and suppresses colitis in mice [Perrier et al., 2013]. Increased levels of TNFα were detected in models of colitis-associated colorectal cancer, and TNF blockade was found to suppress tumor growth in dextran sulfate sodium (DSS) or azoxymethane (AOM)-induced models of colitis-associated colon cancer [Popivanova et al., 2008].

Nevertheless, cytokine blockade, and in particular TNFα blockade, remain a crucial field of interest for IBD therapy. A traditional therapy for IBD includes anti-inflammatory agents such as sulfasalazine, 5-aminosalicylic acid, corticosteroids, azathioprine and infliximab. These medicines are often used in combination and carefully monitored, but do not represent a solution for a long-term cure because of several side effects that alter normal physiological functions by inducing osteoporosis, lipolysis, increased circulating glucose, increased water retention and hypertension, as well as immunosuppression. Anti-TNFα therapy, however, seems to be beneficial only in certain subgroups of patients. This may be due to the complex interplay of diverse cytokines inside inflamed mucosa, as well as to the presence of microbial, genetic and immunological factors. Currently, two monoclonal antibodies against TNFα, Infliximab and Adalimumab, are used in therapy. Clinical trials show that these therapies were effective in patients with CD [Hanauer et al., 2006; Peyrin-Biroulet et al., 2008]. Nevertheless, anti-TNFα drugs often cause side effects, such as GI problems, anaemia, hypersensitivity, and drug intolerance when taken for prolonged time due to the formation of antibodies against-TNFα therapies. Moreover, patients on combination therapy have increased risk of developing lymphoma [Siegel et al., 2009]. In order to reduce their immunogenicity, combination with immunomodulators (thiopurines, cyclosporines) has resulted in significant improvement in response and remission

of IBD, but also in increased risk of infections especially bacterial and fungal [Lichtenstein et al., 2012].

5.5. TACE in intestinal inflammation

About 10% of proteins present on the surface of the cell membrane are cleft proteolytically and released into the extracellular space by a process called "ectodomain shedding" [Fong et al., 2011]. During this process, there is a production of soluble proteins, such as cytokines and their receptors, which can trigger or inhibit autocrine and paracrine signalling pathways. Proteins that appear to be more involved in ectodomain shedding are enzymes belonging to the ADAM family (A disintegrin and metalloproteinase domain), such as ADAM17, also known as TNF-α converting enzyme (TACE), a 70 kDa enzyme stored in perinuclear regions which, following phosphorylation on Thr735, translocates on cell surface [Soond et al., 2005]. ADAM family members are multi-domain I-type transmembrane proteins, consisting of a peptide N-terminal signal sequence, a pro-domain, a metalloproteinase domain corresponding to the catalytic domain of the molecule, a disintegrating domain, an EGF-like domain rich in cysteine, a single transmembrane domain and a cytoplasmic portion (Fig. 20). The pro-domain, blocking the catalytic site, has the function of keeping TACE in its inactive state. It is cut by the proteinase furin, which allows TACE to activate and thus explicate its function [Gonzales et al., 2004; Leonard et al., 2005; Moss et al., 2007]. The metalloproteinase domain is responsible for the enzymatic cut of different substrates present on the cell surface. The disintegrin domain is able to interact with integrins, thus affecting cell adhesion as well as cell-cell interactions [Edwards et al., 2008]. EGF-like domain is involved in the recognition of substrates [Edwards et al., 2008], whereas cytoplasmic domain interacts with several signalling molecules such as protein kinase C (PKC) and ERK [Blobel, 2005].

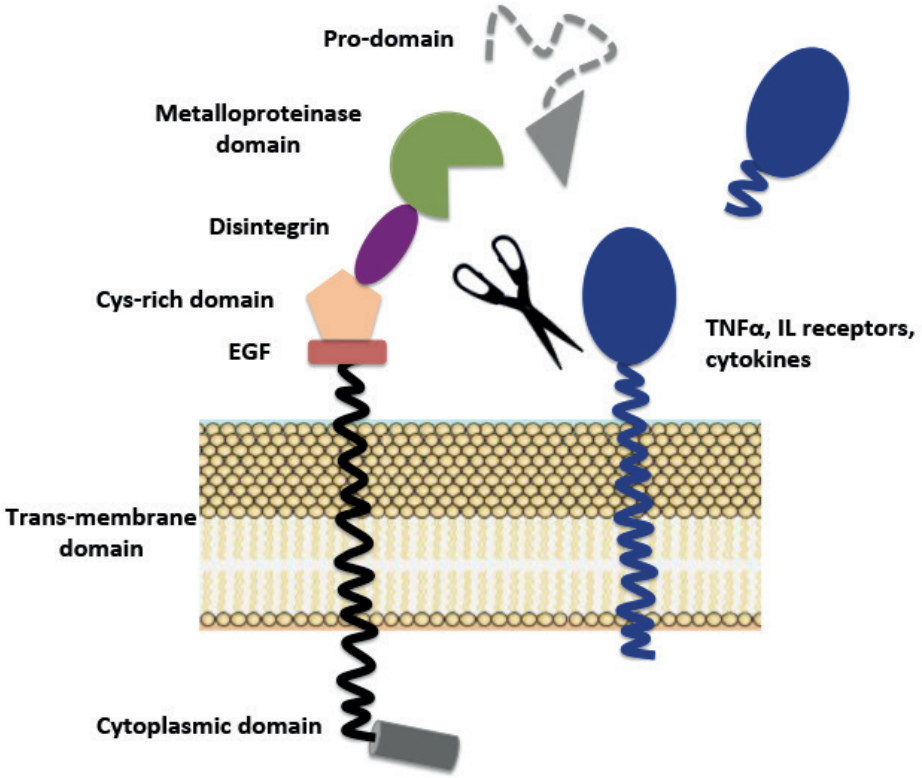


Figure 20. Schematic representation of TACE structure and ectodomain shedding.

TACE can be considered a molecular switch that controls immune response, tissue regeneration and tumor development. It is activated by MAPK p38 that, once activated by oxidative stress, determines the phosphorylation of TACE on Thr735. It is also activated by PKC, oncogenes, 12-myristic 13-acetate and ROS. The presence of ROS induces the formation of disulfide bridges that allow TACE to assume its active conformation and its movement towards the membrane. ROS also mediate up-regulation of TACE expression [Zeng et al., 2013; Fontani et al., 2016].

TACE acts on different substrates, such as growth factors anchored to the membrane surface, cytokines (TNFα, IL-6), cytokine receptors (TNF-R1 and TNF-R2), growth factor receptors (VEGF-R2, ErbB, EGFR), ectoenzymes, cell adhesion molecules (ICAM-1, VCAM-1, NCAM), amyloid precursor protein (APP) and others. The cut of these substrates is of functional importance for their activation, inactivation, modulation and expression [Borrell-Pagès et al., 2003; Lee et al., 2003]. Therefore, an alteration in expression and function of TACE may result in various pathological processes. It is known that inflammation is characterized by

uncontrolled release of pro-inflammatory cytokines such as TNFα. In fact, the membrane-bound TNFα acquires the pro-inflammatory activity after its cleavage by TACE, therefore TACE represents a prerequisite for immune response in various inflammatory diseases [Alexopoulou et al., 2006]. Moreover, it has been speculated that its inhibition could improve autoimmune diseases [Moss et al., 2008]. It is worth noting that the activation of TNFα is also due to other molecules, but *in vivo* TACE is the most effective one and this makes it a possible therapeutic target for TNFα-mediated pathologies.

TACE is normally expressed in the intestinal mucosa, and increases in that of patients with IBD, and its expression is up-regulated by TNFα activity [Brynskov et al., 2002; Bzowska et al., 2004]. Transmigration of polymorphomononuclear leukocytes (PMNL) across epithelia is the primary event of acute phase in CD, and it can be responsible for the TNFα secretion by intestinal cells [Betis et al., 2003]. Moreover, in this phase of acute inflammation, an increase of the expression of TACE, but not of its inhibitor TIMP-3, occurs in intestinal endothelial cells (IEC) [Cesaro et al., 2009], demonstrating the early involvement of TACE in TNFα production during PMNL transepithelial migration. In DSS-induced colitis model, reduced levels of TACE were related to increased sensitivity to colitis [Chalaris et al., 2010]. In fact, TACE significantly increases during the period of DSS treatment and returns to normal levels in the remission phase. Therefore, TACE is important to provide resistance to colitis in rats [Shimoda et al., 2016]. Moreover, the treatment with a specific inhibitor of TACE, compound 11p, reduces clinical signs of DSS-induced colitis maintaining the integrity of colon mucosa and avoiding the infiltration of inflammatory cells [Sharma et al., 2014]. In addition to these findings, it is possible TACE involvement in mucosa repair by inducing epithelial cells proliferation and goblet cell differentiation, due to activation of Epidermal Growth Factor Receptor (EGFR) signalling pathway. There are some contradictory data regarding the link between TACE expression levels and pathogenesis of IBD. In particular, some data show that the TACE expression levels in epithelial cells and in intestinal biopsies derived from intestinal mucosa of IBD patients were not significantly different from control samples [Freour et al., 2009; Monteleone et al., 2012]. On the contrary, other data demonstrate that TACE expression enhances in CD patients and promotes neutrophil migration and colitis [Cesaro et al., 2009].

These differences may be due to different inflammatory conditions of intestinal mucosa and/or therapy used.

TACE can be considered a target in therapies for chronic inflammatory diseases. It has been demonstrated that inhibition of TACE by orally bioavailable small molecular weight molecules, would be more effective than blocking downstream cytokine production [Newton et al., 2001]. Two potential leads for orally bioavailable compounds able to inhibit TACE, such as 2-imidazolidinones and tetrahydropyrimidin-2-(1H)-ones, have been developed [DasGupta et al., 2009].

TACE activity is down-regulated by the natural tissue inhibitor TIMP-3 [Ma et al., 2014], but it has been demonstrated that also Tetraspanin CD9 and ICAM-1 negatively regulate TACE -mediated TNFα shedding on the cell surface [Tsukamoto et al., 2014]. TIMP-3 is present in both uninflamed and inflamed gut mucosa and predominantly expressed in macrophage-like cells or fibroblastic-like cells of *lamina propria*, and in endothelial cells [Vaalamo et al., 1998]. It is essential for innate immunity and inhibits the inflammatory response [Smookler et al., 2006]. In fact, studies based on timp3-/- mice or gene transfer *in vitro*, show that the loss of TIMP-3 causes deregulation of cleavage of TNFα and its receptors by inactivation of TACE in innate immunity. Also, very severe colitis, associated with increased expression of inflammatory cytokines, has been determined in TIMP-3-deficient mice after treatment with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [Freour et al., 2009].

5.6. TACE and other pathologies

In rheumatoid arthritis and osteoarthritis, the super-expression of TNFα involves overproduction of enzymes that degrade both collagen and bone tissue [Brennan et al., 1992; Martel-Pelletier et al., 1999; Firestein, 2003]; the pathogenesis of stroke and multiple sclerosis have been repeatedly associated with inflammation [Stokkers et al., 1995-1996; Hallenbeck, 2002; Planas et al., 2006].

In tumors, however, the observed TACE overexpression was associated with an excessive "shedding" of transmembrane growth factors, which thus become

able to bind their receptors [Blobel, 2005]. This interaction results in the regulation of genes responsible of the proliferation, cell growth, differentiation, migration, adhesion and resistance to cellular apoptosis [Yarden & Sliwkowski, 2001]. In particular, the EGFR pathway is super-activated in a variety of tumor types, such as lung, breast, colon, prostate, pancreas, and ovaries, of which it promotes progression and metastases. Therefore, the use of TACE inhibitors in single therapy or in combination with other anti-EGFR drugs, may be of therapeutic value in the anticancer treatment. In addition, the excessive binding of growth factors to their receptors was also related to the onset of these pathologies. TACE inhibitors could also be used in therapies useful for the prevention of kidney disease [Lautrette et al., 2005].

Several studies on myocardial tissue have linked some cardiovascular diseases such as myocarditis, hypertrophic obstructive cardiomyopathy and dilated cardiomyopathy, with high levels of TACE [Satoh et al., 2000]. Moreover, the overexpression of TACE is also present in patients with refractory asthma and pneumonia [Berry et al., 2006]. Additionally, TACE appears to be involved in metabolic disorders such as diabetes. In fact, the use of TNFα-Protease Inhibitor-1 (TAPI-1) increases insulin sensitivity, resulting in decreased hyperglycaemia and vascular inflammation [Federici et al., 2005]. Up until now, it seems that the increase in TACE is only associated only with the progression of the pathologies. On the contrary, the increase in TACE activity is positive for Alzheimer's disease caused by β-amyloid peptide deposition in the cerebral tissue, following the cut by its transmembrane APP protein, by β- and γ-secretase. This protein alternatively may be cut by TACE and ADAM10, which act within the sequence of the β-amyloid peptide, preventing the formation of the amyloidogenic peptide responsible for generating brain amyloid plaques [Verbeek et al., 1997]. Therefore, TACE has been proposed as a novel anti-Alzheimer therapy [Hooper & Turner, 2002].

5.7. Antioxidants in intestinal oxidative stress

New therapeutic approaches in IBD involve enzymatic and non-enzymatic antioxidants that act the base of pathogenic mechanism suppressing ROS generation. The oldest and the most commonly used therapy is 5-amino salicylic acid, known to block inflammatory effects derived from COX activation and to have ROS scavenging capabilities [Miles & Grisham, 1994].

NAC has been reported to show benefits in both animal model experiments and clinical tests in IBD patients showing an improvement in the inflamed intestinal mucosa [Guijarro et al., 2008]. NAC is able to scavenge free radicals and metal ions involved in ROS increase, and to stimulate GSH synthesis [Kasperczyk et al., 2014]. Besides, it is able to inhibit NF-κB [Haddad, 2002], and increase GSH and catalase activities after long-term oral administration in mouse colitis model [Amrouche-Mekkioui & Djerdjouri, 2012].

Lecithinized human Cu/Zn-SOD has been tested in clinical trials because of its positive effects in IBD treatment due to inhibition of NF-κB activity, exhibiting benefits and safety for 14 day when administered to UC patients [Suzuki et al, 2008; Porfire et al., 2014]. Moreover, a recombinant SOD enzyme has been shown to have a strong antioxidant activity in scavenging ROS in a mouse colitis model [Hou et al., 2014].

Natural compounds used in the antioxidant therapy are polyphenols, as curcumin, resveratrol, quercetin, cathechin, present in plant and in foods of plant origin [Moura et al., 2015]. Also food rich of micronutrients with antioxidant properties, such as α-tocopherol, vitamin E and C, can ameliorate ROS induced symptoms [Seifried et al., 2007]. In particular, polyphenols have shown to possess antioxidant, anti-inflammatory, immunomodulatory and antiapoptotic properties, and therefore have been proposed for prevention and treatment of IBD. They scavenge free radicals, and inhibit NF-κB signalling and MAPK pathways [Romier et al., 2009]. Polyphenols improve intestinal homeostasis by promoting intestinal healthy microbiota, defence systems offered by SOD and GPX, and tight junction stability in the gut, whereas they inhibit the generation of pro-inflammatory cytokines, such as TNFα [Kaulmann & Bohn, 2016]. Epidemiological studies have demonstrated that a vegetarian diet rich in polyphenols is able to lower the risk

and relieve the symptoms of IBD [Chiba et al., 2010]. However, potential adverse effects such as pro-oxidant effect at high doses, and interference with the absorption and metabolism of xenobiotics, are to be taken in consideration.

Among polyphenols, RE is an oldie but goodie. In some human studies, it has been shown to decrease inflammation with no side effects [Zamora-Ros et al., 2012; Tomé-Carneiro et al., 2013]. However, no human studies are known so far that demonstrate the anti-inflammatory effect of RE in IBD. The supplementation of RE decreases oxidative stress and reduces inflammation by down-regulating the p38 MAPK, activated by TNFα and recognized as critical in pathogenesis of IBD [Docena et al., 2010]. It is also able to inhibit NF-κB and increase GSH and SOD levels in mice with DSS-induced colitis [Youn et al., 2009; Yao et al., 2010]. Studies are not concordant over RE influence on NO levels, but many of they claim that RE diminishes iNOS expression and NO production [Chan et al., 2000; Larrosa et al., 2009]. RE is able to influence intestinal microbiota homeostasis by reducing bacteria overgrowth, increasing *Lactobacilli* and *Bifidobacteria*, and limiting bacterial infiltration in sub-epithelial tissues during inflammation [Larrosa et al., 2009]. RE administration reduces to normal levels the excess of Th cells, involved in intestinal inflammation, including markers for neutrophil infiltration, ICAM-1 and VCAM-1 [Cui et al., 2010]. Based on current literature, RE could potentially be useful for acute phases of IBD and could substitute anti-inflammatory drugs currently used in therapy of IBD. Considering that RE is rapidly metabolized and inactivated, derivatives with more powerful antioxidant and anti-inflammatory properties have been synthesized (Fig. 21). Selma et al. have demonstrated the efficacy of synthetic glucosyl, glucosylacyl and glucuronide resveratrol derivatives in inhibition of cytokine production caused by foodborne microbial pathogen infections. Another group demonstrated that these modifications allow RE to exert dual effect: prevent its rapid metabolism and reduce mucosa barrier imbalance responsible of diarrhea [Larrosa et al., 2010].

Moreover, 1,2,4-oxadiazole analogues of RE have shown prominent antioxidant and anti-inflammatory potential [Gobec et al., 2015].

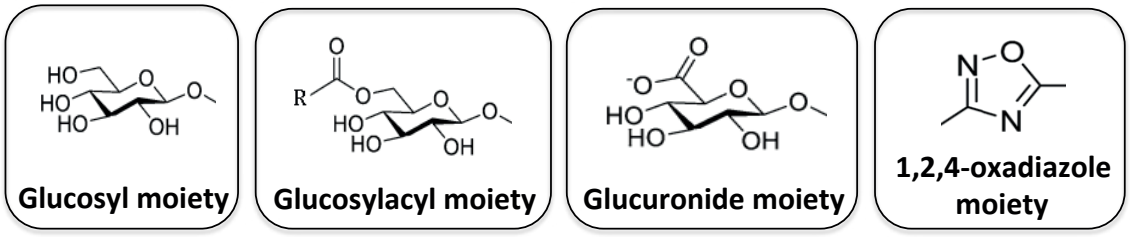


Figure 21. Moieties of resveratrol synthetic derivatives.

Some drugs already present for treating diseases different than IBD have been shown to be useful for ameliorating oxidative stress and intestinal inflammation in IBD. In particular, it has been reported that Telmisartan, an inhibitor of angiotensin II type I, is able to reduce ROS generation and inflammatory responses in IBD patients, by suppressing NF-κB, lipid peroxidation, iNOS and COX-2 expression, whereas it increases GSH and SOD levels in a rat model of colitis [Arab et al., 2014]. SOD activity results increased also in a rat model of colitis after treatment with inhibitors of hydroxymethylglutaryl-CoA reductase, Simvastatin and Rosuvastatin [Maheshwari et al., 2015].

AIM OF THE THESIS

Free radicals are common outcome of normal aerobic cellular metabolism and endogenous antioxidant system plays its decisive role in prevention of excessive amount of free radicals. However, imbalanced defence mechanism of antioxidants, overproduction or incorporation of free radicals from environment to living system leads to various pathological states. Given the involvement of oxidative stress and antioxidants in bone remodelling alterations and inflammatory bowel diseases, the aim of this thesis was to study: 1) the role and molecular mechanisms of 17β-E2 and natural antioxidants on stress induced apoptosis in osteocytes; 2) the antioxidant ability of RE and of its benzoselenophene derivatives, VD0, VD1 and VD2, in restoring the physiological redox state in both osteocytes and intestinal myofibroblasts; 3) the role of oxidative stress and TNFα on the expression and activation of TNF-α converting enzyme (TACE) involved in chronic intestinal inflammation.

Regarding the first point, the molecular mechanisms by which 17β-E2 prevents apoptosis and the abnormal expression and release of factors related to increased oxidative stress and bone remodelling in osteocytes, the regulatory cells in bone metabolism, were studied. In fact, 17β-E2 deficiency associated to menopause, can cause excessive bone loss related to microdamage, increased oxidative stress and osteocyte apoptosis observed in osteoporosis. In particular, the effect of 17β-E2 on ROS production and the activation of redox-regulated kinases, such as extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK), were also studied. Indeed, in osteocytes, these kinases are involved in oxidative stress-induced apoptosis and expression of factors related to bone remodelling, such as receptor activator κB ligand (RANKL), osteoprotegerin (OPG) and sclerostin [Fontani et al., 2015]. To clarify the molecular action of estrogen on these events, the role of mitogen-activated protein kinase phosphatase-1 (MKP-1) and glutathione S-transferase P1-1 (GSTP1-1), both involved in the regulation of JNK activity, were investigated [Adler et al., 1999; Carlson et al., 2009; Takeuchi et al., 2009; Laborde et al., 2010].

The role of blueberry juice (BJ) obtained from *Vaccinium myrtillus* berries, native in Italian Apennines, on ROS production, apoptosis and expression of osteoclastogenic factors in osteocytes, was investigated and compared to that of dry extracts of BB (BE), GT (GTE) and HP (HE), being the last two known for their powerful antioxidant properties and beneficial effects in reducing the bone mass loss. Indeed, high concentration of phenolic compounds, including anthocyanins, in the *Vaccinium myrtillus* berry promoted this fruit as "functional food" in human diet [Rao et al., 2008; Prencipe et al., 2014; Ancillotti et al., 2016]; moreover, polyphenols have potent antioxidant properties and also a preventive role against postmenopausal osteoporosis [Ishimi, 2006; Rao et al., 2008]. Some studies indicate the ability of blueberries in improving the balance between bone formation and bone resorption [Zhang et al., 2013; Li et al., 2014].

Regarding the second point, the antioxidant ability of RE benzoselenophene derivatives, VD0, VD1 and VD2, in restoring the physiological redox state, has been investigated in both osteocytes and intestinal myofibroblasts and compared to RE effect. In fact, RE, differently to that occurs for its derivatives, once absorbed is rapidly metabolized thus leading to the reduction of its effects [Larrosa et al., 2010; Selma et al., 2012].

Regarding the third point, the role of oxidative stress and TNFα on the expression and activation of TACE, has been investigated in a cellular line of human colonic myofibroblasts. Previous findings in these cells have shown that oxidative stress and pro-inflammatory cytokines, such as TNFα, are both related to an increased expression of ICAM-1 and release of sICAM-1 by a TACE proteolytic cleavage [Fontani et al., 2016]. High levels of ICAM-1 and sICAM-1 have been found in mucosa and serum of patients with IBD [Nielsen et al., 1994; Ghosh & Panaccione, 2010]. Indeed, high levels of sICAM-1, detected in serum of CD patients, probably derive from the cleavage of ICAM-1 from cell surface within gut mucosa [Nielsen et al 1994].

These studies are part of a wide field of research that concerns the identification of redox regulated-substrates and -biological processes involved in oxidative stress-related pathologies, and the characterization of natural and synthetic compounds with antioxidant properties that may be useful for new dietary or pharmacological approaches.

These studies were performed in a murine osteocyte-like cell line, MLO-Y4, and a human colon mucosa myofibroblasts, CCD-18Co (18Co). MLO-Y4 cells have a similar phenotype and many characteristics of mature osteocytes [Kato et al., 1997], and therefore represent a good *in vitro* model to study osteocyte apoptosis subsequent to microdamage and bone disease [Kalajzic et al., 2013]. These cells control bone remodelling process and mineralization, the reason for which osteocytes are involved in the repair of microdamage and microfractures [Verborgt et al., 2000; Henriksen et al., 2009]. In MLO-Y4 cells, serum starvation induces an increase of ROS levels and this mimics an *in vivo* condition similar to that occurs after microdamage or estrogen deprivation [Kalajzic et al., 2013; Fontani et al., 2015; Domazetovic et al., 2017]. In fact, apoptosis due to microdamage may be related to a disruption of blood and fluid flow with consequent lack of various endocrine factors, including estrogens [Tami et al., 2002; Bakker et al., 2004; Al-Dujaili et al., 2011]. Excessive oxidative stress occurring during various physio-pathological conditions has been related to elevated turnover of bone remodelling process and subsequent bone mass loss, in part due to abnormal apoptosis of osteocytes [Finkel et al., 2000; Weinstein et al., 2000; Noble, 2005; Banfi et al., 2008; Jilka et al., 2013]. Indeed, oxidative stress affects bone cell differentiation and inhibits bone mineralization process [Baek et al., 2010; Filaire & Toumi, 2012; Romagnoli et al., 2013; Fontani et al., 2015].

For the studies on intestinal myofibroblasts, 18Co cells, with many properties similar to those of intestinal subepithelial myofibroblasts, were used as a model in which oxidative stress was induced by using TNFα or BSO in the presence or not of NAC. Indeed, in 18Co cells, 25 μM BSO induces an increase of ROS levels similar to that present in intestinal subepithelial myofibroblasts isolated from inflamed mucosa of CD patients [Fontani et al., 2014]. In fact, the presence of oxidative stress can be responsible of an excessive and uncontrolled inflammation as occurs in CD [Laroux et al., 2001; Romagnoli et al., 2013a; Fontani et al., 2016].

MATERIALS AND METHODS

1. Reagents

BIOCARE Medical (Pacheco, California, USA)	Negative control mouse IgG.
Bio-Rad (Hercules, California, USA)	Chemiluminescence reagent kit.
Cell Signaling Technology (Beverly, Massachusetts, USA)	Anti-caspase-3, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK, anti-JNK, anti-TACE antibodies.
GE Healthcare (Little Chalfont, Great Britain)	Polyvinylidene difluoride (PVDF) membrane.
Invitrogen (Carlsbad, California, USA):	2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), Lipofectamine RNAiMAX, Trypan blue exclusion test.
MyBioSource (San Diego, California, USA)	Anti-TACE-phospho-Thr735 antibody.
R&D Systems (Minneapolis, Minnesota, USA)	OPG and RANKL quantitative sandwich enzyme immunoassay kits.
Roche Laboratories (Nutley, New Jersey, USA)	Cell Death detection ELISA plus kit.
Santa Cruz Biotechnology (Dallas, Texas, USA)	Protein A/G PLUS-Agarose immunoprecipitation reagent, anti-β-Actin, anti-GSTP1-1, anti-OPG, anti-RANKL antibodies.

Sigma-Aldrich (Saint Louis Missouri, USA)	Minimum Essential Medium Eagle, L-glutamine, sodium bicarbonate, non-essential amino acids, sodium pyruvate, dimethyl sulfoxide (DMSO), 17β-estradiol (17βE2), 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), buthionine sulfoximine (BSO), diphenyleneiodonium chloride (DPI), N-acetylcisteine (NAC), 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl-triphenylphosphonium chloride monohydrate (Mito TEMPO), tumor necrosis factor- alpha (TNFα), trypsin, Tris/HCL, Triton X-100, NaCl, NaF, Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), GSTP1-1 siRNA, scr siRNA, protease and phosphatase inhibitor cocktail, Folin-Ciocalteu, gallic acid, Laemmli's SDS sample buffer.
Thermo Fisher Scientific (Waltham, Massachusetts, USA)	Pierce BCA protein assay kit, bovine serum albumin.

2. Cell cultures and treatments

2.1. MLO-Y4 cell culture and treatment

MLO-Y4 osteocyte-like cells were a gift from Dr. Lynda Bonewald, University of Missouri-Kansas City. MLO-Y4 were cultured at 37 °C in a 5% CO_2 humidified atmosphere in α-MEM medium supplemented with 5% Calf Serum, 5% Fetal Bovine Serum, 2 mM L-glutamine. Culture media was supplemented with 72 mg/L penicillin and 100 mg/ml streptomycin.

- *Experiments with 17β-E2:*

Cells at 70–80% confluence were treated for 30 min in a medium, in which FBS was substituted with charcoal stripped FBS, with 17-β-E2 at various concentrations (1, 10 or 100 nm), or in complete medium in the absence (untreated cells) or in the presence of 5 mm NAC for 16 h. Subsequently, after removal of the media, treated cells were cultured for another 4 or 24 h in serum free medium in the presence of 17-β-E2 or NAC. Untreated cells were cultured for 4 or 24 h in serum free medium (S, starved cells) or in fresh complete medium (C, control). 0.01% ethanol (vehicle for estrogen) was added to all untreated 17-β-E2 cells. For experiments with inhibitors, cells were pretreated for 30 min in complete medium with 1μm DPI or 100 μm AEBSF or 5 nm Mito TEMPO, in the presence or in the absence of 17β-E2. Subsequently, the complete medium was removed and for another 4 or 24 h, cells were cultured with inhibitors with or without estrogen in serum-free medium. In experiments with DPI, 0.003% DMSO was added to DPI untreated cells.

- *Experiments with extracts of BB, GT and HP*:

Dry extracts of BB (BE), GT (GTE) and HP (HE) were obtained by Aboca and BB juice (BJ) was obtained by frozen BB harvested in August 2016 in Tuscany Apennines in which *Vaccinium myrtillus*, among the various species of blueberry, is the native species predominantly present. Freshly frozen blueberries from Tuscan Apennines (aliquots of 100 g) were homogenized in a Waring blander, filtered under vacuum and centrifuged (20000 g for 10 min) to obtain BB juice (BJ), aliquots of BJ were stored at -20°C. Experiments were performed in MLO-Y4 cells at 70–80% confluence treated for 60 min in a medium, in the presence or not of

aliquots of BE, GTE, HE and BJ containing various concentrations of total soluble polyphenol fraction (TSP), from 15 μg/mL to 100 μg/mL, measured as subsequently described. Treated cells after removal of the media, were cultured for another 4 or 24 h in serum free medium in the presence of extracts or BJ Untreated cells were cultured for 4 or 24 h in serum free medium (S, starved cells) or in fresh complete medium (C, control). 0.01% final concentration of DMSO was present in controls and in all treated and untreated cells.

- *Experiments with RE benzoselenophene derivatives:*

Cells at 70–80% confluence were starved for 24 h in order to induce the oxidative stress. Subsequently, starved MLO-Y4 were treated or not for other 3 h (short-term study) or 24 h (long-term study) with various concentrations (2.5-200 μM) of RE and its derivatives (VD0, VD1 and VD2). Other experiments were performed adding RE and its derivatives during the induction of oxidative stress (prevention study), in particular 1 h prior to 24 h-starved MLO-Y4. Unstarved MLO-Y4 were considered controls. 0.008% final concentration of DMSO was present in controls and in all treated and untreated cells.

- *MLO-Y4 transfection*

Transfection of GSTP1-1 siRNA duplexes corresponding to two DNA target sequences of mouse GSTP1-1 (5′-CCCUCAUCUACACCAACUA[dT][dT]-3′) and (5′-UAGUUGGUGUAGAUGAGGG[dT][dT]-3′) or scrambled siRNA (scr siRNA) (Universal Negative Control #1), was performed in cells grown in 6-well plates with 150.000 cells per well. Lipofectamine RNAiMAX was incubated with SiRNA in α-MEM without serum and antibiotics at room temperature for 20 min, then the lipid/RNA complexes were combined with cells with gentle agitation to a final concentration of 60 nM in α-MEM containing fetal bovine serum. After 24 h, medium was changed and cells were treated as described above. The ability of siRNA to silence the expression level of GSTP1-1 mRNA was checked 24 h after transfection by western blot analysis.

2.3. 18Co cell culture and treatment

CCD-18Co (18Co) cells, obtained from American Type Culture Collection (Manassas, VA), were used in our experiments with PDL=27-36, given that the line begins to senescence at about PDL=42. 18Co cells were cultured at 37 C in a 5% CO_2 humidified atmosphere in Minimum Essential Medium Eagle with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. Culture media was supplemented with 72 mg/L penicillin and 100 mg/ml streptomycin.

- *Experiments with RE benzoselenophene derivatives:*

18Co cells were serum starved at confluence for 24 h and treated or not during the last 16 h with 25 μM BSO treated or not for other 3 h (short-term study) or 24 h (long-term study) with various concentrations (2.5-200 μM) of RE and its derivatives (VD0, VD1 and VD2). Other experiments were performed adding RE and its derivatives during the induction of oxidative stress (prevention study), in particular 1 h prior to BOSO treatment. 24 h-starved 18Co were considered controls. 0.008% final concentration of DMSO was present in controls and in all treated and untreated cells.

- *Experiments for TACE:*

18Co cells, serum starved at confluence for 24 h and treated or not during the last 16 h with 25 μM BSO or 20 mM NAC or NAC + BSO, were stimulated or not for other 24 h with 1 or 10 ng/ml TNFα. Additional experiments were performed in 24 h serum starved 18Co cells treated or not, during the last 16 h, with BSO in the presence or absence of 10μM DPI. In experiments with DPI, 0.003% DMSO (vehicle for DPI) was added in the respective DPI untreated cells. Subsequently these cells were stimulated for 24 h with TNFα.

3. Performed assays

3.1. Intracellular ROS production assay

The intracellular levels of ROS were assayed in MLO-Y4 and 18Co seeded in 12-well plates and treated as described above. 30 min before the end of the various treatments, 5 g/L of H_2DCFDA was added in culture medium. This substance is cell-permeant and a chemically reduced form of fluorescein used as an indicator for reactive oxygen species in cells. After PBS washing, cells in well plates were lysed in RIPA buffer (50 mM Tris/HCL pH 7.5, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, phosphatase and protease inhibitor cocktail), centrifuged at 14.000 rpm (ALC PK121R, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 10 minutes, and analysed immediately by florescence spectrophotometric analysis at 510 nm. Data have been normalized on total protein and expressed as fold-increase over the control values or as percentage of ROS production compared to control.

3.2. Apoptosis assay in MLO-Y4 cells

MLO-Y4 seeded in 6-well plates at the density of 30,000 cells per cm^2 and treated as described above, were used to assess apoptosis using Cell Death Detection ELISA plus Kit according to manufacturer's instructions. Data are expressed as fold-increase over the control values of mono- and oligo-nucleosomes obtained from 10^4 cells using the following formula: absorbance of the samples/absorbance of the corresponding control values.

3.3. Total soluble phenolic content

TSP of BJ, BE, GTE and HP were determined using Folin-Ciocalteu reagent, following the method described by Singleton and Rossi with slight modifications. Test samples of 10 µL each of solubilized extract or BJ were mixed with 10 µL of water and 100 µL of Folin-Ciocalteu's phenol reagent (1:10). After 5 min, 80 µL of saturated sodium carbonate solution (7.5 w/v in water) was added to the mixture. The mixture was kept for 2 h in the dark at room temperature, after which the

absorbance was measured at 765 nm. Gallic acid was used as a reference standard, and TSP was expressed as mg gallic acid equivalents/mL of extract or juice.

3.4. Benzoselenophene derivatives

RE benzoselenophene derivatives VD0, VD1 and VD2 were obtained by direct selenylation of RE with $SeCl_2$ generated in situ by Se and SO_2Cl_2 in dry tetrahydrofuran [Tanini et al. 2015]. Modulation of Se/SO_2Cl_2/RE stoichiometry led to the formation of mixtures with different amounts of parent benzoselenophene (VD0), monochloro- (VD1) and dichloro- (VD2) benzoselenophene derivatives (Fig. 5).

3.5. Cytotoxicity assay

The cytotoxicity of RE and its benzoselenophene derivatives has been detected using Trypan blue exclusion test, in 24 h-starved 18Co or in unstarved MLO-Y4 treated or not for 24 h with RE or derivatives at the respective highest concentrations used, 50 μM for 18Co cells and 200 μM for MLO-Y4.

3.6. Western blot analysis

The phosphorylation of ERK1/2 and JNK, the activation of caspase-3, the expression of RANKL, OPG, sclerostin, GSTP1-1, MKP-1 and ADAM17 were performed by Western blot in MLO-Y4 and 18Co seeded in p60 plates treated and stimulated or not as described for each experiment. Cells were lysed for 30 min at 4 °C in RIPA buffer containing 50 mM Tris/HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, containing protease and phosphatase inhibitor cocktails, and centrifuged at 11.600 g for 10 min at 4 °C. Equal amounts of total proteins (30–60 μg) from whole-cell extract were suspended in Laemmli's SDS sample buffer and subjected under reducing conditions to SDS/PAGE on 10% gel and electrotransferred to PVDF membrane.

Proteins were visualized by incubating the membranes overnight with specific primary antibodies: anti-caspase 3 or anti-phospho-ERK 1/2 or anti-phospho-JNK, or anti-RANKL or anti-OPG or anti-sclerostin or anti-GSTP1-1 or anti-MKP-1. For the detection of ADAM17 and ADAM17 phosphorylated at Thr735, the samples

were lyophilized and 100 mg of proteins were subjected to SDS/PAGE on 10% (w/v) gel. PVDF membranes were probed with anti-ADAM17 or anti-β-actin. Subsequently, membranes were stripped by incubation for 30 min at 50°C in buffer containing 62.5 mM Tris/HCl, pH 6.7, 100 nM 2-mercaptoethanol, 2% SDS and, after extensive washing, the membranes were reprobed with anti-ERK1/2 or anti-JNK or anti-β-actin or anti-ADAM17-phospho-Thr735. β-actin bands were used for normalization and densitometric analysis. Secondary antibodies conjugated to horseradish peroxidase were used to detect antigen-antibody complexes with a chemiluminescence reagent kit.

Densitometric analyses of the western blot bands were performed using Amersham Imager 600 (GE Healthcare, Little Chalfont, UK) and ImageJ software (http://imagej.nih.gov/ij/). Graphical representations were created using Microsoft Excel and GraphPad Prism 6 (GraphPad software, San Diego, California, USA). Values shown in the graphs are means ± SEM of three or six independent experiments performed in triplicate, and are reported as fold increase over control set al 1 or as percentage of control.

3.7. Immunoprecipitation and immunoblot

Immunoprecipitation was carried out using 2 μg of anti-GSTP1-1 or anti-JNK antibody or negative control mouse IgG and 400 μg of total protein lysates, for 16 h at 4 °C. Subsequently, protein A/G PLUS-Agarose Immunoprecipitation Reagent was added (20 μL) for 1 h at 4–5 °C, after which washes were carried out in PBS supplemented with Tween-100 (0.5%). Precipitate was suspended in sample buffer without β-mercaptoethanol (nonreducing conditions) and was subjected to SDS/PAGE (10%) followed by electrotransfer to PVDF membrane. Reaction with the anti-JNK or anti-GSTP1-1 overnight at 4 °C was carried out. Subsequently to stripping, membranes were reprobed with anti-GSTP1-1 or anti-JNK or anti β–Actin.

3.8. RANKL and OPG release measurement in MLO-Y4

Receptor activator κB ligand and OPG release was measured in the culture medium of MLO-Y4 seeded in twelve-well plates at the density of 20,000 cells per cm^2 treated for 24 h as described above. RANKL and OPG assays were performed in cell culture medium using quantitative sandwich enzyme immunoassay kits, according to the manufacturer's instructions. Data, normalized on total protein content, were expressed as fold-increase over the control values.

3.9. Protein assay

Protein concentration was determined by Pierce bicinhoninic acid solution (BCA) protein assay kit using bovine serum albumin as the standard.

3.10. Statistical analysis

Results were expressed as means ± S.E.M. of three or more experiments performed in triplicate. Statistical analysis was performed using Student's t test, one-way and two-way ANOVA followed by Bonferroni's multiple comparison test. P values ≤0.05 were considered statistically significant**.**

RESULTS

1. Results I

1.1. Estrogen inhibits starvation-induced apoptosis in osteocytes by a redox-independent process involving association of JNK and glutathione S-transferase P1-1.

Domazetovic V, Fontani F, Marcucci G, Iantomasi T, Brandi ML, Vincenzini MT

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Paper in appendix

Fig. 22A shows that apoptosis increased remarkably after 4 and 24 h from starvation, and it was reduced by about 60% at both times after 30 min pre-treatment with various concentrations of 17-β-E2, as compared to the respective untreated starved cells. The maximum effect of apoptosis inhibition was obtained already with 10 nM 17β-E2, a concentration close to the physiological one in women, subsequently used in the following experiments. The anti-apoptotic effect of 17β-E2 was confirmed in the experiments with caspase-3, an enzyme involved in apoptosis of osteocytes. 17β-E2 reduces also the active form of caspase-3 (17 kDa) obtained by proteolytic cleavage of inactive 32-kDa pro-caspase-3 (Fig. 22B).

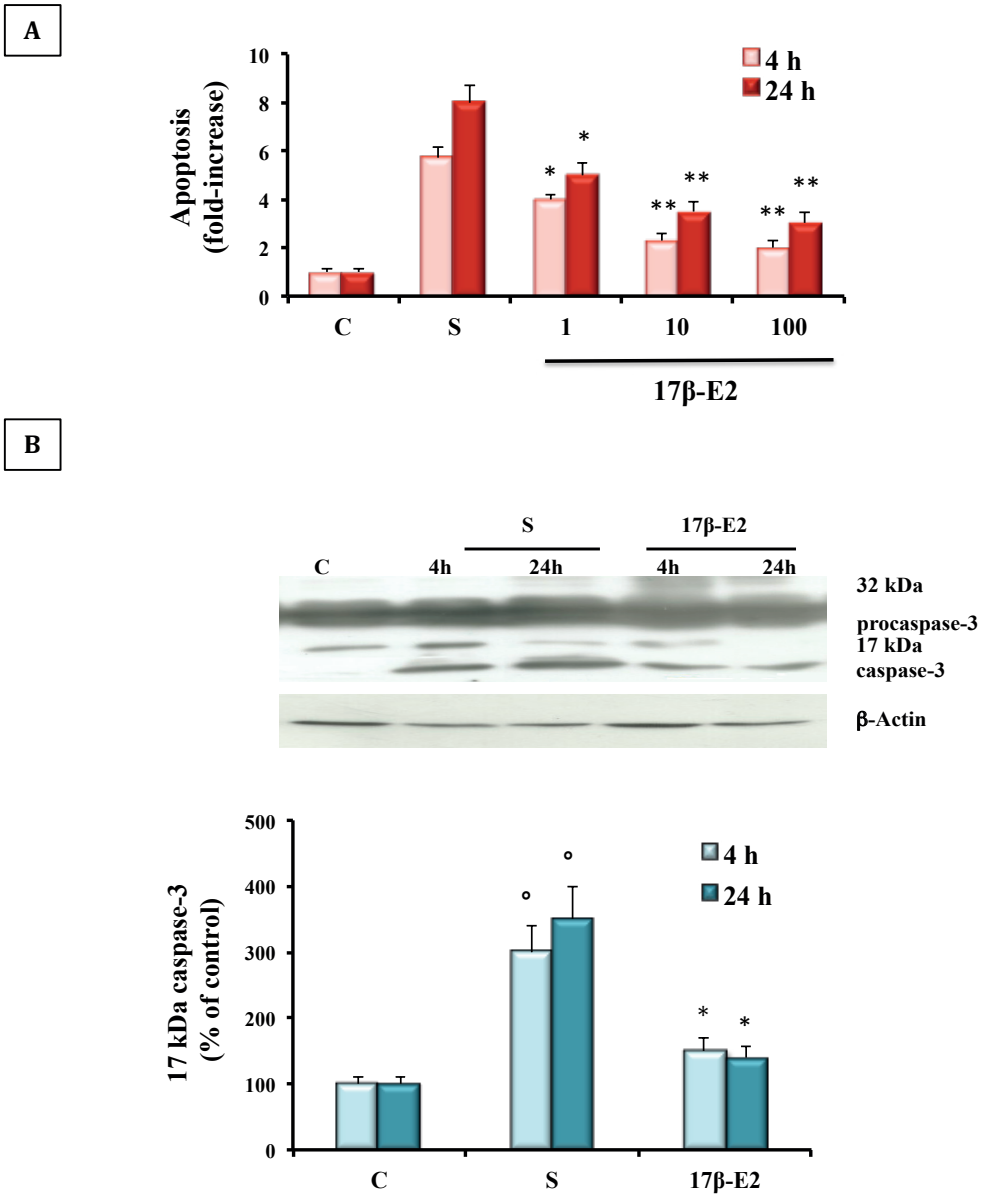

Figure 22. Effect of various concentrations of 17β-E2 on apoptosis and active 17 KDa caspase-3 in MLO-Y4 cells. Apoptosis (A) and active 17 KDa caspase-3 (B) were measured in MLO-Y4 cells cultured for 4 h and 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 17β-E2 as reported in Materials and Methods. Apoptosis data, relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells treated with various concentrations (nM) of 17β-E2, are expressed as fold-increase over the respective C values and are the mean ± SEM of four experiments. Active 17 kDa caspase- 3 was measured in cells treated with 10 nM 17β-E2 by Western blot analysis. Blots are representative of four experiments. The active 17 kDa caspase-3 values, normalized with β-Actin bands obtained by densitometric analysis, were reported as the mean percentage ± SEM relative to the respective C values in the bottom; $^{*}P \leq 0.05$ and $^{**}P \leq 0.005$ compared to the respective S values; $^{\circ}P \leq 0.005$ compared to the respective C values.

Given that starvation induces ROS both after 4 and 24 h, the variation of ROS levels related to 17β-E2 anti-apoptotic effect, was studied. Fig. 23A shows that 17β-E2 reduces ROS only by 30% after 24 h, as compared to untreated starved cells. In the same figure, the limited 17β-E2 effect on ROS production is compared with that of DPI and AEBSF, both inhibitors of NADPH oxidase, MitoTEMPO, a specific scavenger of mitochondrial ROS, as well as with a direct antioxidant, NAC. The inhibitors were used at concentrations able to inhibit ROS production. The inhibitors of NADPH oxidase showed similar effect to that of 17β-E2, reducing ROS by 40% after 24 h, whereas mitochondrial and direct ROS scavengers showed major antioxidant effect, inhibiting ROS by about 50-60% at both times. Fig. 23B reports the apoptosis levels measured in cells treated with inhibitors and stimulated or not by 17β-E2. No difference in apoptosis in 17β-E2-, DPI- and AEBSF-treated cells at both studied times, and no synergic effect in cells treated with 17β-E2 + inhibitors, were obtained. Differently, MitoTEMPO and NAC decreased apoptosis by about 60% as compared to starved estrogen-treated or untreated cells.

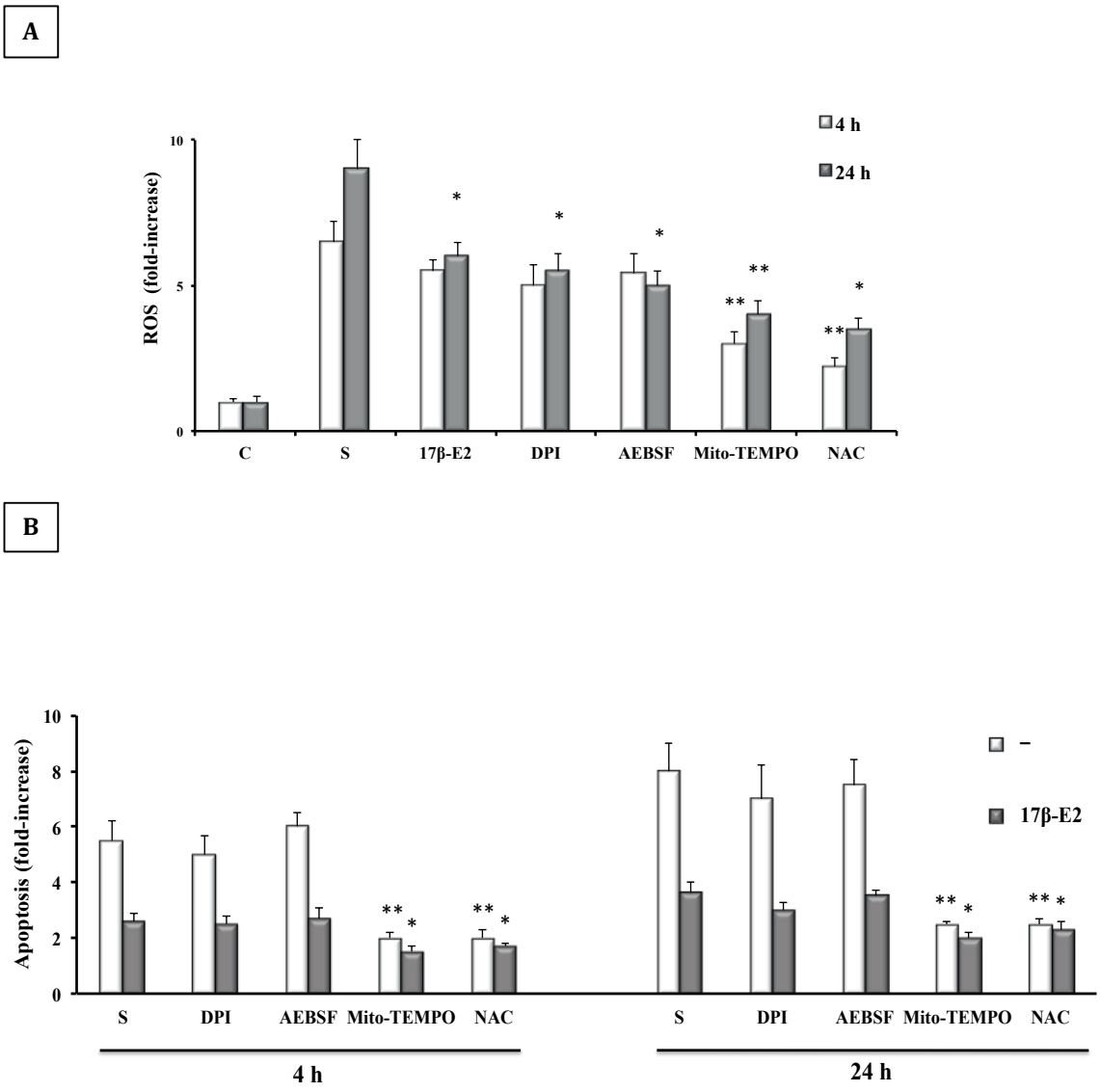

Figure 23. Effect of 17β-E2, DPI, AEBSF, Mito-Tempo and NAC on intracellular ROS and apoptosis in MLO-Y4 cells. Intracellular ROS (A) and apoptosis (B) were measured in MLO-Y4 cells cultured for 4 h and 24 h in complete medium (C, control) or in serum-free medium (S, starved cells) treated or not with 10 nM 17β-E2, 1 μM DPI, 100 μM AEBSF, 5 nM Mito-TEMPO, or 5 mM NAC as reported in Materials and Methods. ROS data, normalized on total protein content, and apoptosis data, relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells, are expressed as fold-increase over the respective C values. The data are the mean ± SEM of four independent experiments; $^{*}P \leq 0.05$ and $^{**}P \leq 0.005$ compared to the respective S values.

Further on, we investigated the effect of 17β-E2 on ERK1/2 and JNK activation, both activated by ROS and involved in regulation of osteogenic factors [Fontani et al., 2015]. In fact, ERK1/2 and JNK phosphorylation increased after 4 and 24 h from starvation, and 17β-E2 inhibited JNK activation at both times by 60-70%, as compared with unstimulated starved cells (Fig. 24A), whereas it had no significant effect on ERK1/2 activation (Fig. 24B). Being JNK particularly involved in starvation-induced apoptosis [Fontani et al., 2015], this represents a further confirmation of the anti-apoptotic effect of 17β-E2.

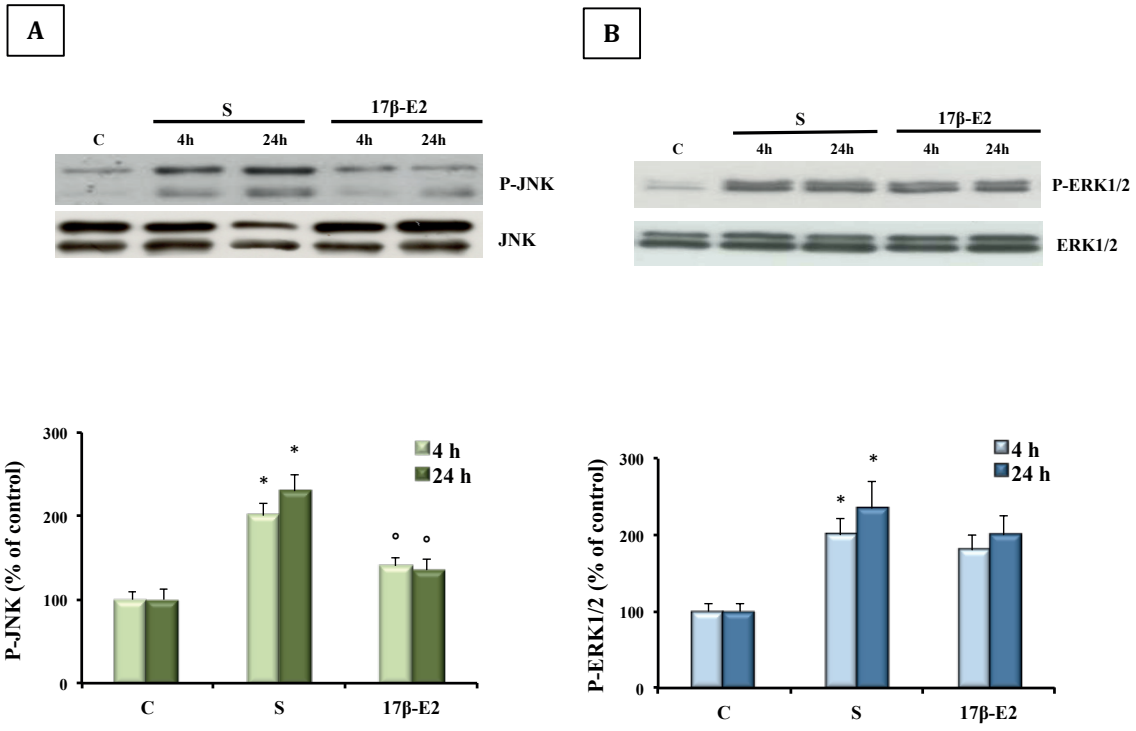

Figure 24. Effect of 17β-E2 on MAPKs phosphorylation in MLO-4Y cells. Phosphorylation of JNK (A) and ERK1/2 (B) were detected by western blot analysis in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2 as reported in Materials and Methods. Blots are representative of four experiments. The values, normalized with JNK or ERK1/2 bands obtained by densitometric analysis, are reported as mean percentage ± SEM relative to the respective C values in the bottom. *P ≤ 0.05 compared to the respective C values; °P ≤ 0.05 as compared to the respective S values.

In order to clarify the involvement of JNK in 17β-E2 anti-apoptotic effect, we evaluated the expression of a specific JNK phosphatase, MKP-1 [Takeuchi et al., 2009], during starvation and after 17β-E2 treatment. In Fig. 25 no difference in expression of MKP-1 at both times was observed, in both 17β-E2-treated and untreated cells.

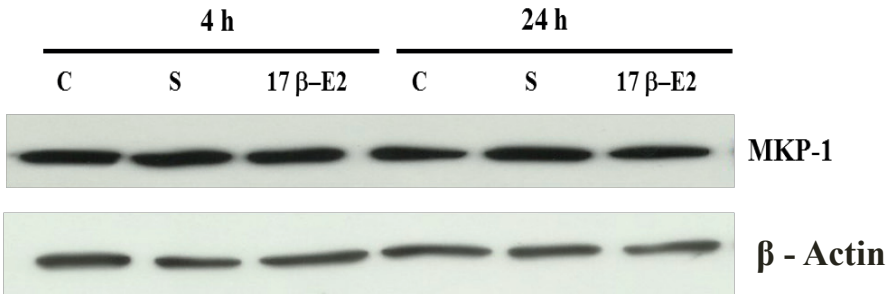

Figure 25. Effect of 17β-E2 on MKP-1 expression in MLO-4Y cells. MKP-1 expression was detected by western blot analysis in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2 as reported in Materials and Methods. Blots are representative of four experiments.

Previous studies demonstrated that monomeric GSTP1-1 binds JNK, inhibiting its activity, and that this complex is sensitive to intracellular oxidative state and/or to GSTP1-1 expression levels [Bernardini et al., 2000; Okamura et al., 2015]. In Fig. 26A no band of JNK or GSTP1-1 or β-Actin was detected in Western blot analysis of immunoprecipitates performed with IgG (negative control) in control cells. Differently, JNK and GSTP1-1 and no β-Actin were detected after immunoprecipitation with anti- GSTP1-1 or anti-JNK antibody (Fig. 26A). These data demonstrate the absence of non-specific bands under these experimental conditions. Fig. 26B shows JNK bands obtained by Western blot analysis under non-reducing SDS/PAGE of immunoprecipitates performed with anti-GSTP1-1 antibody. A decrease in JNK associated with monomeric GSTP1-1 form (26 kD) was registered only in starved cells as compared to control. On the contrary, both 17β-E2 and NAC treatments prevented this dissociation after 4 and 24 h, and the band density of JNK was similar to those of the respective controls (Fig. 26B). The stripping and reprobing of the same blots by anti-GSTP1-1 antibody show that the band density of GSTP1-1 monomeric form changed similarly to that of JNK bands (Fig. 26B). The same results with regard to the association of monomeric form of GSTP1-1 with JNK were obtained by JNK immunoprecipitation experiments performed under non-reducing SDS/PAGE (Fig. 26C). To evaluate the expression of total GSTP1-1 in 17β-E2- and NAC-treated cells, Western blot analysis of cellular lysates in reducing conditions was performed (Fig. 26D). A significant increase in total GSTP1-1 expression in estrogen-treated cells, as compared to control, was measured but no variation was noted in starved and NAC-treated cells.

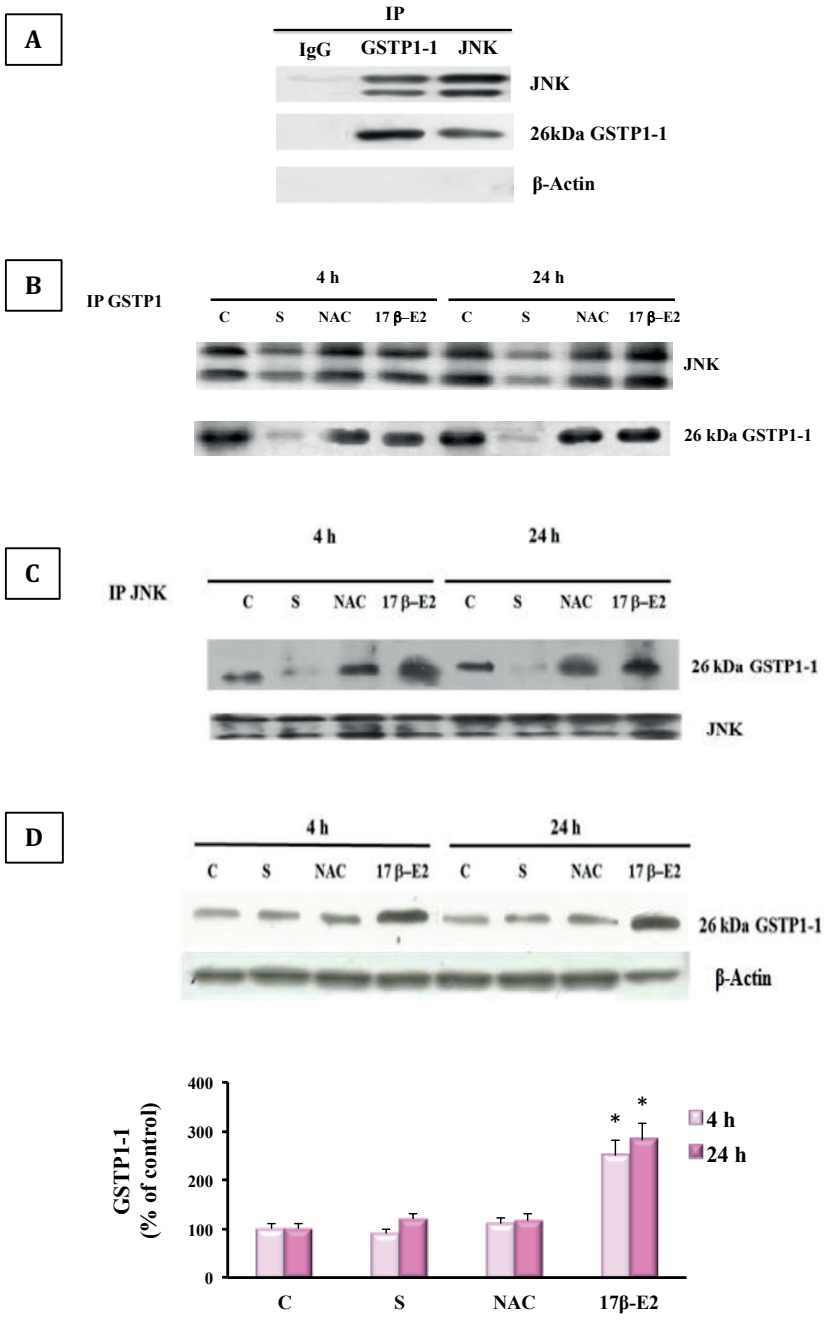

Figure 26. Effect of 17β-E2 and NAC on JNK association with monomeric GSTP1-1 form and GSTP1-1 expression in MLO-4Y cells. JNK association with GSTP1-1 and GSTP1-1 expression were detected in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10nM 17β-E2 or 5mM NAC as reported in Materials and Methods. The negative control was performed in MLO-Y4 cells cultured for 24 h in complete medium by immunoprecipitation with negative control mouse IgG (A). For detection of JNK bound to GSTP1-1 or IgG (A and B), or GSTP1-1 bound to JNK or IgG (A and C), or β-Actin bound to IgG (A) all immunoprecipitates of equal proteins (400 μg) were performed using anti-GSTP1-1 antibody or anti-JNK antibody or IgG respectively, as reported in Materials and Methods. Western blot analyses were performed under non-reducing conditions (without β-mercaptoethanol) and membranes were probed with anti-JNK (A and B) or anti-GSTP1-1 (C) antibodies and subsequently, after stripping, with anti-GSTP1-1 (A and B) or anti-JNK (C) or anti- β-Actin (A) antibodies, respectively. GSTP1-1 expression (D) was detected by Western blot analysis performed under reducing condition. The normalized values with β-Actin bands obtained by densitometric analysis are reported as mean percentage ± SEM relative to the respective C values in the bottom. The blots are representative of four experiments; $^{*}P \leq 0.05$ compared to the respective C values.

In order to confirm GSTP1-1 involvement in JNK regulation, GSTP1-1 silencing was performed. Fig. 27A shows that GSTP1-1 expression decreased in control cells transfected with specific siRNA after 24 h of transfection. Fig. 27B shows that the down-regulation of GSTP1-1 induced a significant increase in apoptosis in starved cells treated with NAC or 17β-E2 at both studied times, as compared to the respective scr siRNA-starved cell values. In particular, down-regulation of GSTP1-1 in 17β-E2-treated cells reverted the apoptosis and JNK activation levels to the values measured in untreated scr siRNA starved cells (Fig. 27B–D). In NAC-treated cells, down-regulation of GSTP1-1 increased also apoptosis and JNK activation levels, but these did not reach the levels of untreated scr siRNA starved cell, even if the changes in JNK activation were not significant as compared with untreated scr siRNA-starved cells (Fig. 27B–D).

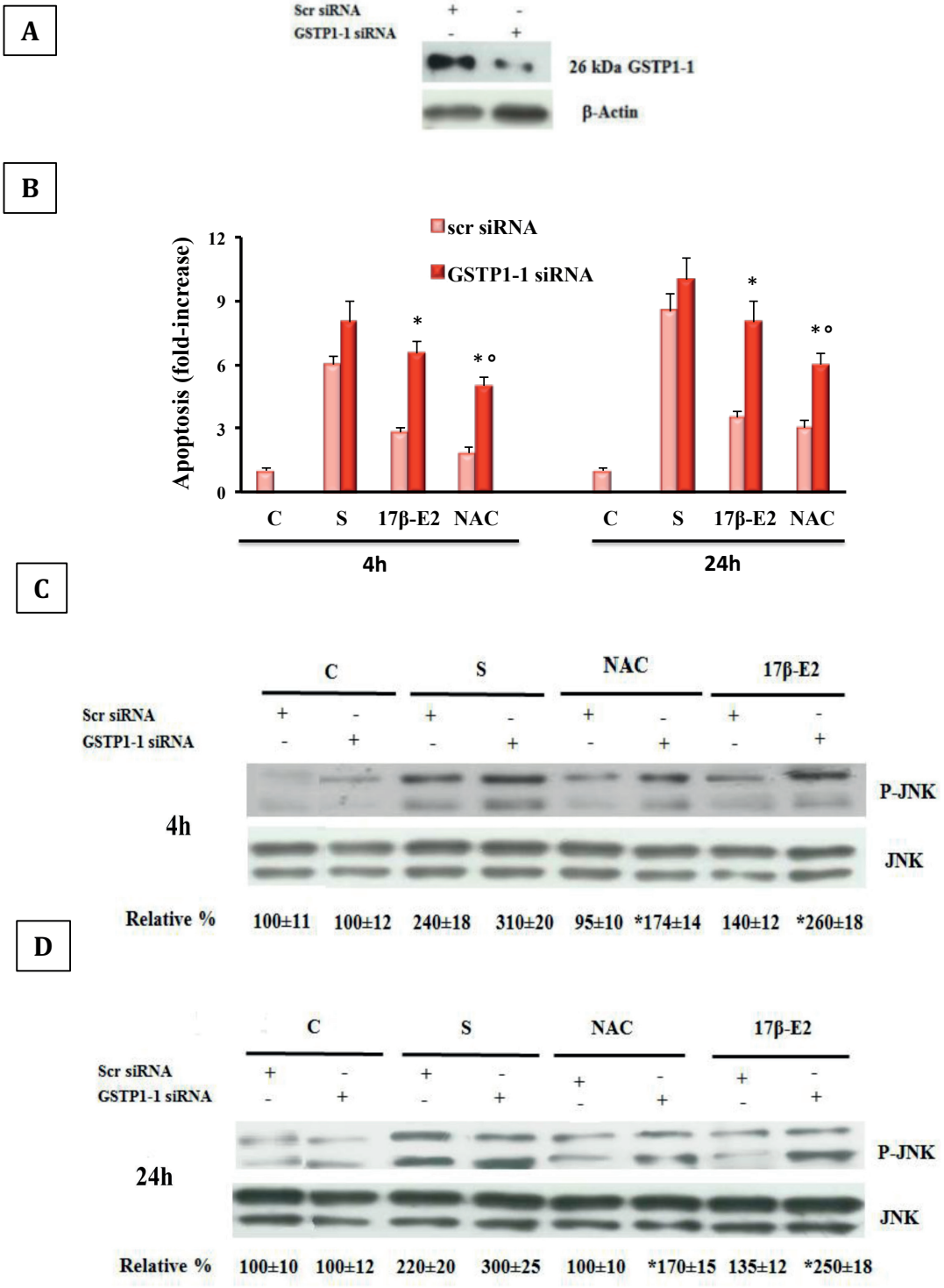

Figure 27. Effect of GSTP1-1 siRNA on apoptosis and JNK phosphorylation in 17β- E2- or NAC-treated MLO-4Y cells. MLO-Y4 cells, transfected with GSTP1-1 siRNA or scr siRNA (negative control) for 24 h, were cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). Transfected S were treated or not with 10nM 17β-E2 or 5mM NAC as reported in Materials and Methods. GSTP1-1 expression (A) and JNK phosphorylation after 4 h (C) or 24 h (D) were detected by western blot analysis. The blots are representative of three experiments and the normalized values with JNK bands obtained by densitometric analysis are reported as mean percentage ± SEM relative to the respective C values. Apoptosis data (B), relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells, are expressed as fold-increase over C values, and are the mean ± SEM of three independent experiments; *P ≤ 0.05 compared to the respective scr siRNA-treated cell values. °P ≤ 0.05 compared to scr siRNA S values.

Fig. 28 summarizes the possible effects of 17β-E2 and NAC on the association of JNK to GSTP1-1 in relation to JNK and apoptosis activation in starved MLO-4Y cells.

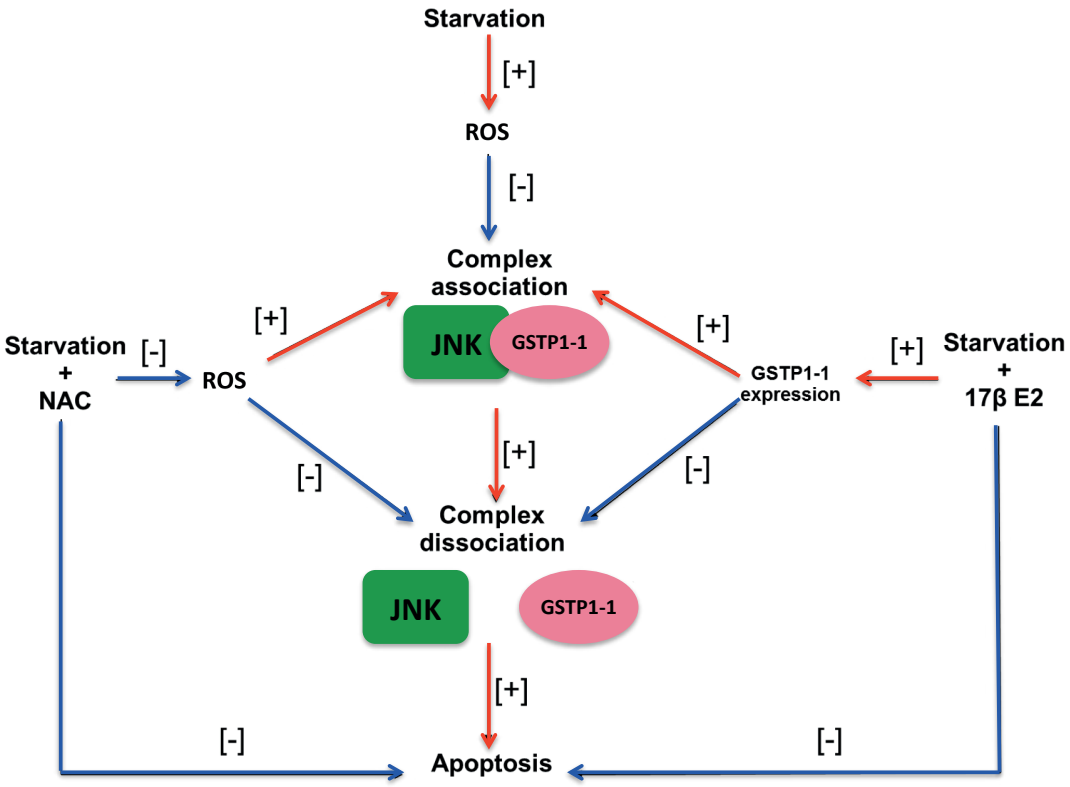

Figure 28. Summary of 17β-E2 and NAC different mechanisms on JNK-GSTP1-1 complex formation in starved MLO-Y4 cells. NAC and 17β-E2 reduce JNK-GSTP1-1 complex dissociation- and starvation-induced apoptosis favouring JNK/GSTP1-1 complex association by ROS decrease and GSTP1-1 expression increase, respectively [+] = increase; [-] = decrease.

Considering that in MLO-Y4 cells a relationship among apoptosis, increased oxidative state and expression of RANKL, OPG and sclerostin has been previously found [Fontani et al., 2015], the expression of these factors was evaluated. In particular, in starved MLO-Y4 cells oxidative stress-induced activation of JNK and ERK1/2 was related to RANKL and sclerostin up-regulation, whereas only JNK activation was involved in decreased OPG levels. Fig. 28A,B shows after 4 or 24 h of starvation a significant increase of RANKL and sclerostin levels, as compared to control, which decreased by about 50%-60% after 17β-E2 treatment without returning to control values. On the contrary, OPG remarkably decreased at both starvation times, as compared to control cells, and 17β-E2 inhibited the reduction of OPG expression by about 50% only after 24 h of starvation (Fig. 28C). Also in this case, 17β-E2 was not able to revert OPG levels to control value (Fig. 28C). Fig. 28D shows that 17β-E2 treatment significantly lowered RANKL release after 24 h of starvation as compared with untreated and starved cells. However, 17β-E2 did not restore RANKL release to control values in accordance to that observed on RANKL expression. Similar results were obtained after 4 h of starvation (data not shown). Regarding OPG protein release, we were not able to measure the levels of this protein by ELISA kit in MLO-Y4 in agreement with previously reported results [Al-Dujaili et al., 2011; Fontani et al., 2015]. This may be due to the low OPG levels below the minimum threshold of the assay method and/or to the high levels of RANKL present in these cells. In fact, high levels of RANKL could interfere with the assay of OPG, as reported in the instructions of the ELISA kit. For this reason, the RANKL/OPG ratio was measured by the expression values obtained in Western blot analysis (Fig. 28A,C). The ratio increased in a similar manner after 4 h and 24 h of starvation as compared to control cells (Fig. 28E), and 17β-E2 significantly lowered the ratio values at both times by about 40–70%, respectively (Fig. 28E). The decrease in ratio value after 24 h of starvation was significantly higher than that obtained after 4 h.

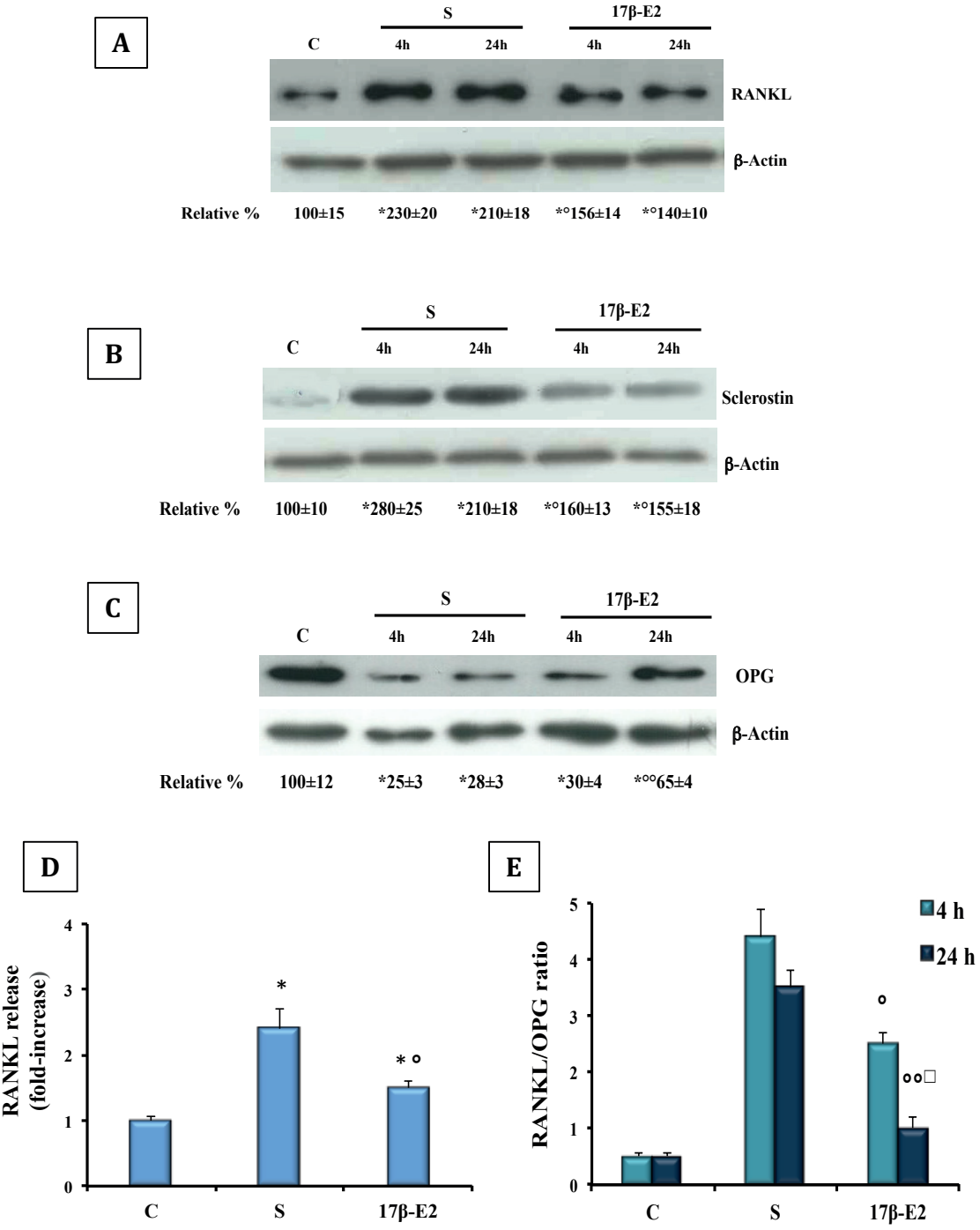

Figure 28. Effect of 17β-E2 on RANKL, sclerostin and OPG expression, RANKL release and RANKL/OPG ratio in MLO-Y4 cells. RANKL, sclerostin, and OPG expression and RANKL release were measured in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2 as reported in Materials and Methods. RANKL (A), sclerostin (B), and OPG (C) were measured by western blot analyses of cell lysates. Blots are representative of four experiments and the normalized values with β-Actin bands obtained by densitometric analysis are reported as mean percentage ±SEM relative to the respective C values. RANKL release (D) was detected by immunoenzymatic method in 24 h culture medium, and the values are expressed as fold-increase over the control values. (E) RANKL/OPG ratio was obtained by the expression values measured with western blots. The data are the mean ±SEM of four independent experiments; $^{*}P \leq 0.05$ compared to the respective C values; $^{\circ}P \leq 0.05$ and $^{\circ\circ}P \leq 0.005$ compared to the respective S values; $^{\square}P \leq 0.005$ compared to S values for 4 h and stimulated with 17β-E2.

1.2. Role of blueberry preparations in oxidative stress-induced apoptosis of osteocytes.

(Unpublished data)

The antioxidant effect of various concentrations of total soluble polyphenol (TSP) fraction of BJ (15 µg/mL - 100 µg/mL) was measured after 4 h and 24 h of treatment in starved osteocytes. Fig. 29 shows a concentration-dependent reduction of ROS levels at both times. Lower TSP concentrations of the juice (15-25 µg/mL) reduced significantly ROS levels of about 25% as compared to starved cells (S), whereas higher concentrations (50-100 µg/mL) caused further ROS decrease reaching about 60%.

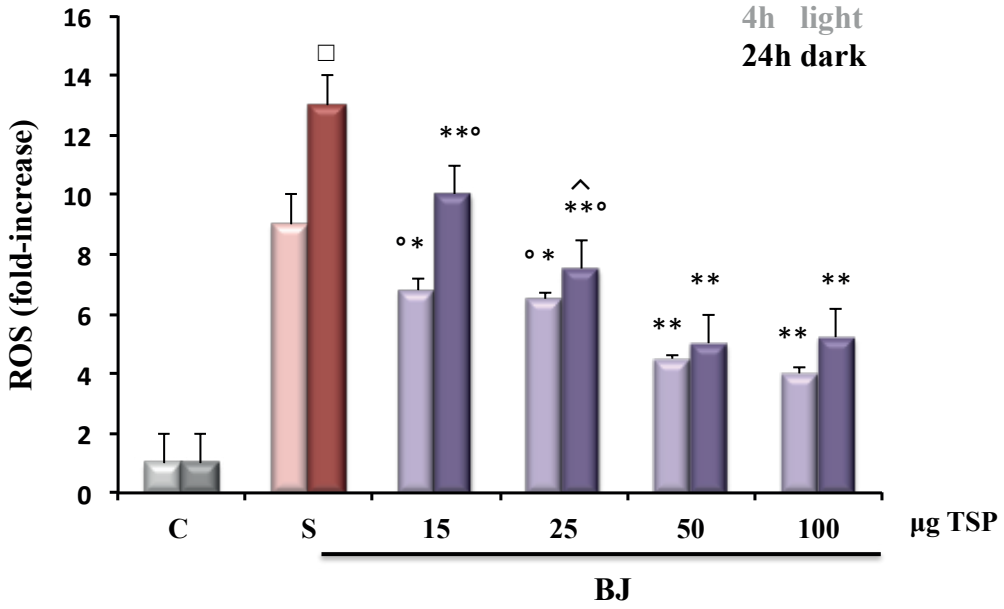

Figure 29. Effect of BJ on intracellular ROS in MLO-Y4 cells. Intracellular ROS were measured in MLO-Y4 cells cultured for 60 min in a medium in the presence of various µg of TSP of BJ and then for another 4 h and 24 h in complete medium (C, control) or in serum-free medium (S, starved cells) treated or not with BJ as reported in Materials and Methods. ROS data, normalized on total protein content, are expressed as fold-increase over the respective C values. The data are the mean ± SEM of four independent experiments; $^{*}P \leq 0.05$ and $^{**}P \leq 0.001$ compared to the respective S values at 4h and 24 h; $^{\circ}P \leq 0.05$ compared to the respective 50 and 100 µg TSP; $^{\wedge}P \leq 0.05$ compared to the respective 15 µg TSP at 24 h; $^{\square}P \leq 0.001$ compared to the respective S values at 4 h.

Subsequently, BJ antioxidant effect was compared to that of BE, GTE and HE after 4 h and 24 h (Fig. 30) by using 50 µg/mL TSP, considering that at this concentration BJ already induced the maximum decrease of ROS. Fig. 30 shows that no difference was measured between BJ and BE, but the decrease of ROS levels in both treatments was significantly less in comparison to GTE and HE which were able to induce ROS decrease of about 80% at both times.

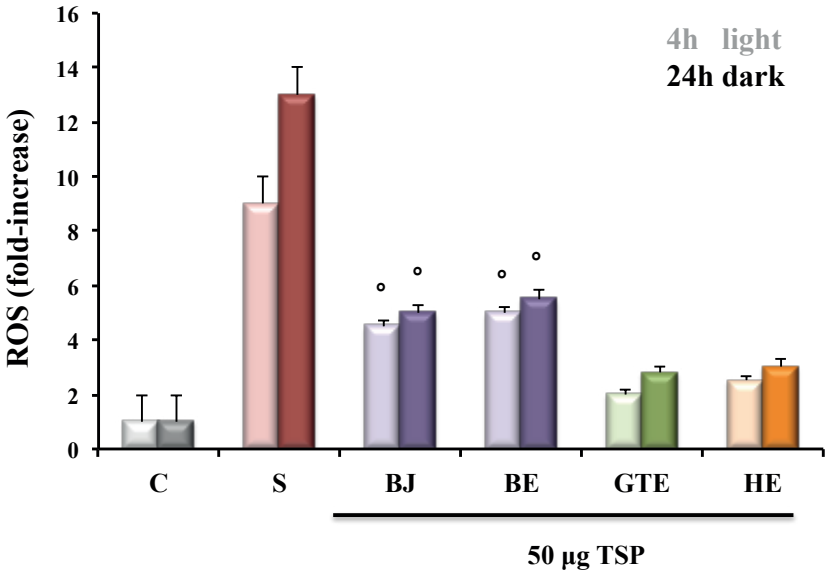

Figure 30. Comparison of BJ effect on intracellular ROS with that of BE, GTE and HE in MLO-Y4 cells. Intracellular ROS were measured in MLO-Y4 cells cultured for 60 min in the presence of 50 µg TSP of BJ, BE, GTE and HE and then for 4 h and 24 h in complete medium (C, control) or in serum-free medium (S, starved cells) in the presence or not of the same treatments as reported in Materials and Methods. ROS data, normalized on total protein content, are expressed as fold-increase over the respective C values. The data are the mean ± SEM of four independent experiments; °P ≤ 0.05 compared to the respective GTE and HE.

The antiapoptotic effect was also measured in starved cells after 24 h of treatment with 50 μg/ml TSP of BJ and extracts; all treatments inhibited similarly the apoptosis of about 70% - 90% as compared to starved cells (Fig. 31).

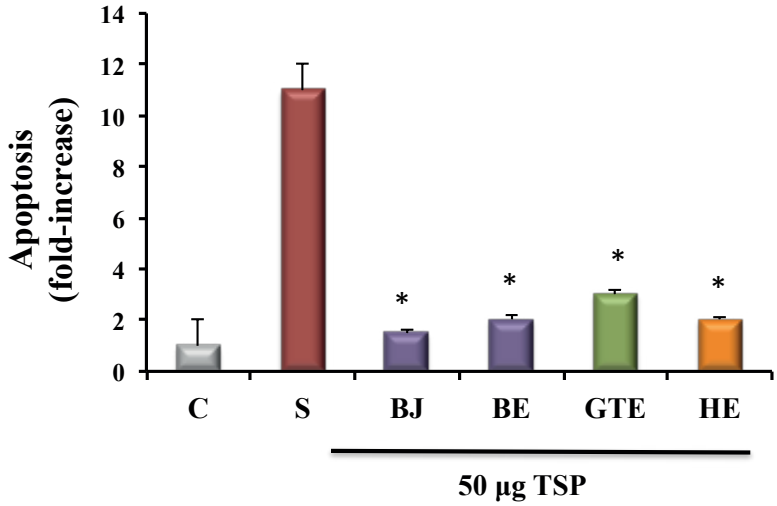

Figure 31. Comparison of BJ effect on apoptosis with that of BE, GTE and HE in MLO-Y4 cells. Apoptosis was measured in MLO-Y4 cells cultured for 60 min in the presence of 50 μg of TSP of BJ, BE, GTE and HE and then cultured for 24 h in complete medium (C, control) or in serum-free medium (S, starved cells) in presence or not of the same treatments as reported in Materials and Methods. Apoptosis data, relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells, are expressed as fold-increase over C values and are the mean ± SEM of four experiments; $^*P \leq 0.05$ compared to S values.

BJ and extracts were also able to inhibit oxidative stress-induced expression of osteoclastogenic factors such as RANKL and sclerostin (Fig. 32).

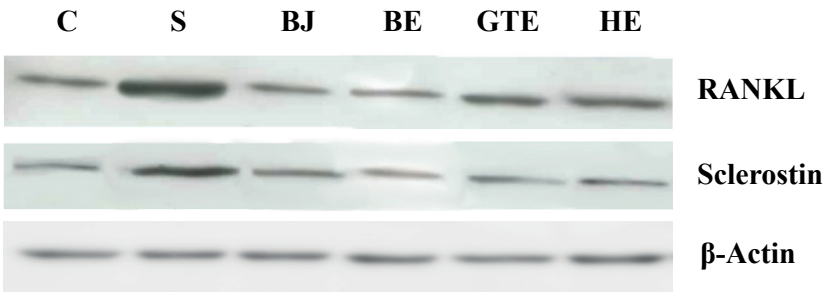

Figure 32. Effect of BJ on RANKL and sclerostin expression in MLO-Y4 cells. RANKL and sclerostin expression were measured in MLO-Y4 cells cultured for 60 min in the presence of 50 μg of TSP of BJ, BE, GTE and HE and then cultured for 24 h in complete medium (C, control) or in serum-free medium (S, starved cells) in presence or not of the same treatments as reported in Materials and Methods. Expression levels were measured by western blot analyses of cell lysates. Blots are representative of four experiments.

2. Results II

2.1. Protective role of benzoselenophene derivatives of resveratrol on the induced oxidative stress in intestinal myofibroblasts and osteocytes.

Domazetovic V, Fontani F, Tanini D, D'Esopo V, Viglianisi C, Marcucci G, PanzellaL, Napolitano A, Brandi ML, Capperucci A, Menichetti S, Vincenzini MT, Iantomasi T

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Paper in appendix

Firstly, the cell viability was evaluated in 18Co and MLO-Y4 treated whit RE or its benzoselenophene derivatives at the respective highest concentrations used, 50 μM for 18Co and 200 μM for MLO-Y4. Cell viability did not significantly change in the presence of all compounds as compared to that of the respective untreated cells (Fig. 33A and B). Therefore, considering the lack of cytotoxic effects, RE and its derivatives were used at these or at lower concentrations in further experiments.

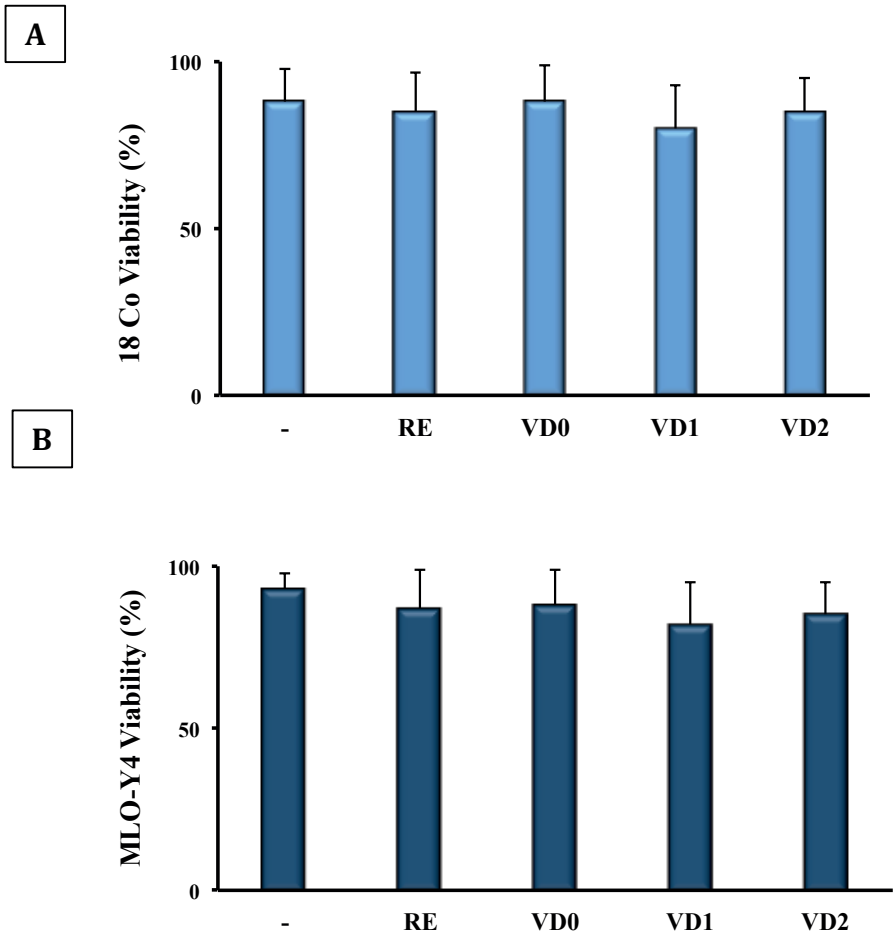

Figure 33. Cytotoxicity of RE and benzoselenophene derivatives. Starved 18Co (A) or unstarved MLO-Y4 (B) were treated for 24 h or not with RE or VD0, VD1, VD2 derivatives at 50 µM or 200 µM, respectively. Cell viability was performed by Trypan blue exclusion test and the values, expressed as percentage of total cells (living and dead cells), are the mean ± SEM of six experiments in triplicate.

Subsequently, ROS production was measured in the absence or in the presence of various concentrations (2.5-50 μM) of RE or its benzoselenophene derivatives in BSO-treated 18Co cells. In 18Co cells BSO treatment induced a significant increase in ROS production of about 60% in the short-term study (3 h) (Fig. 34A) and of about 100% in long-term study (24 h), as compared to control (Fig. 34B). Fig. 34A shows that the behaviour of ROS production following the short-term treatment with RE and its derivatives was mainly concentration-dependent. RE at 10 μM reduced significantly ROS levels restoring them to control values, whereas the same effect was obtained already with 5 μM VD0 and VD1. The highest concentration used of RE and its derivatives (50 μM) decreased significantly ROS levels below those of control (Fig. 34A). This effect was major in derivative-treated cells, even if not significant. An evident concentration-dependence was not obtained in long-term study performed in BSO-treated 18Co cells in the presence of RE and its derivatives (Fig. 34B). In these conditions, among all the studied concentrations of RE, only 50 μM was able to reduce ROS levels restoring them to control values (Fig. 34B). On the contrary, this effect was evident already at the lowest concentration of derivatives (2.5 μM), whereas 50 μM VD0, VD1 and VD2 decreased remarkably ROS levels as compared to control and 50 μM RE. Fig. 34B shows also that ROS production detected with 10 μM VD1 was significantly lower than that of control values, differently to that occurred for VD0 and VD2.

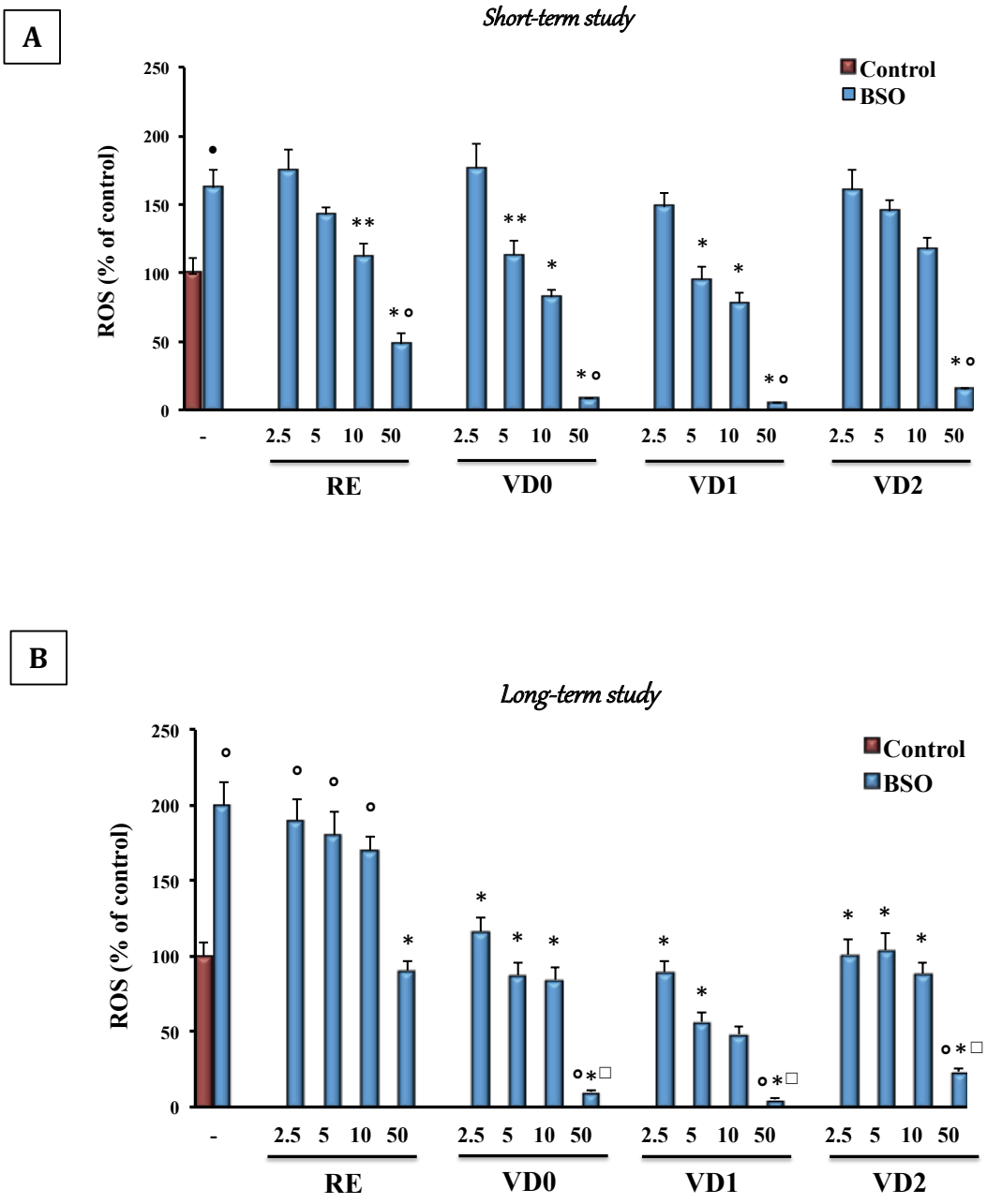

Figure 34. Intracellular ROS production in BSO-treated 18Co cells after short- and long-term treatment with RE and benzoselenophene derivatives. BSO-treated 18Co cells were treated or not with various concentrations (μM) of RE or VD0, VD1, VD2 derivatives for 3 h (short-term, A) or 24 h (long-term, B) as reported in Materials and Methods. The intracellular ROS production was detected by measuring the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percentage of control (BSO-, RE-and derivatives-untreated cells), are the mean ± SEM of six experiments repeated in triplicate. °P ≤ 0.001 compared to the control 18Co cells; •P ≤ 0.05 compared to the control 18Co cells; *P ≤ 0.001 compared to BSO-treated 18Co cells; **P ≤ 0.05 compared to BSO-treated 18Co cells; □P ≤ 0.001 compared to the respective RE-treated 18Co cells.

Starvation induced a remarkable oxidative stress in MLO-Y4 as indicated by ROS production that increased of about 1300% in the short-term study (Fig. 35A) and of about 2200% in the long-term study as compared to control (Fig. 35B). Considering this high oxidative stress, we used RE and its benzoselenophene derivatives at higher concentrations ranging from 25 to 200 μM compared to those used in BSO-treated 18Co. Also in starved-MLO-4Y the decrease of ROS levels was mainly concentration-dependent after the short-term treatment with RE and its derivatives. All compounds at all concentrations significantly reduced starvation-induced oxidative stress in MLO-Y4 cells (Fig. 35A). In particular, RE and its derivatives VD0 and VD2 reduced ROS values in nearly the same way, as compared to starved and untreated MLO-4Y cells, and no significant difference was detected at concentrations of 100 and 200 μM of these derivatives as compared to control values. On the contrary, VD1 restored ROS to control levels at concentrations ranging from 25 to 200 μM (Fig. 35A).

To better compare quantitatively the effect of RE and its derivatives, we performed short-term study treating MLO-Y4 cells with lower concentrations. Fig. 35A shows the lowest concentrations that reduced significantly ROS levels: 10 μM for RE, VD0 and VD2 and 2.5 μM for VD1. Furthermore, among derivatives only VD1 at all concentrations reduced significantly ROS levels as compared to the respective RE concentrations. In the long-term study only 100 and 200 μM RE reduced significantly ROS production, as compared to starved-MLO-Y4. It is to note that the value obtained by treating cells with 200 μM RE was approximately similar to values measured with 50 μM benzoselenophene derivatives (Fig. 35B). VD0, VD1 and VD2 at all concentrations used decreased ROS production, as compared to starved MLO-Y4. The trend of this reduction was concentration-dependent, and ROS levels was significantly lower than that measured in cells treated with the respective concentrations of RE. However, VD1 and VD2, at all concentrations used, did not restore ROS levels to control values, differently to that occurred with 200 μM VD0 (Fig. 35B).

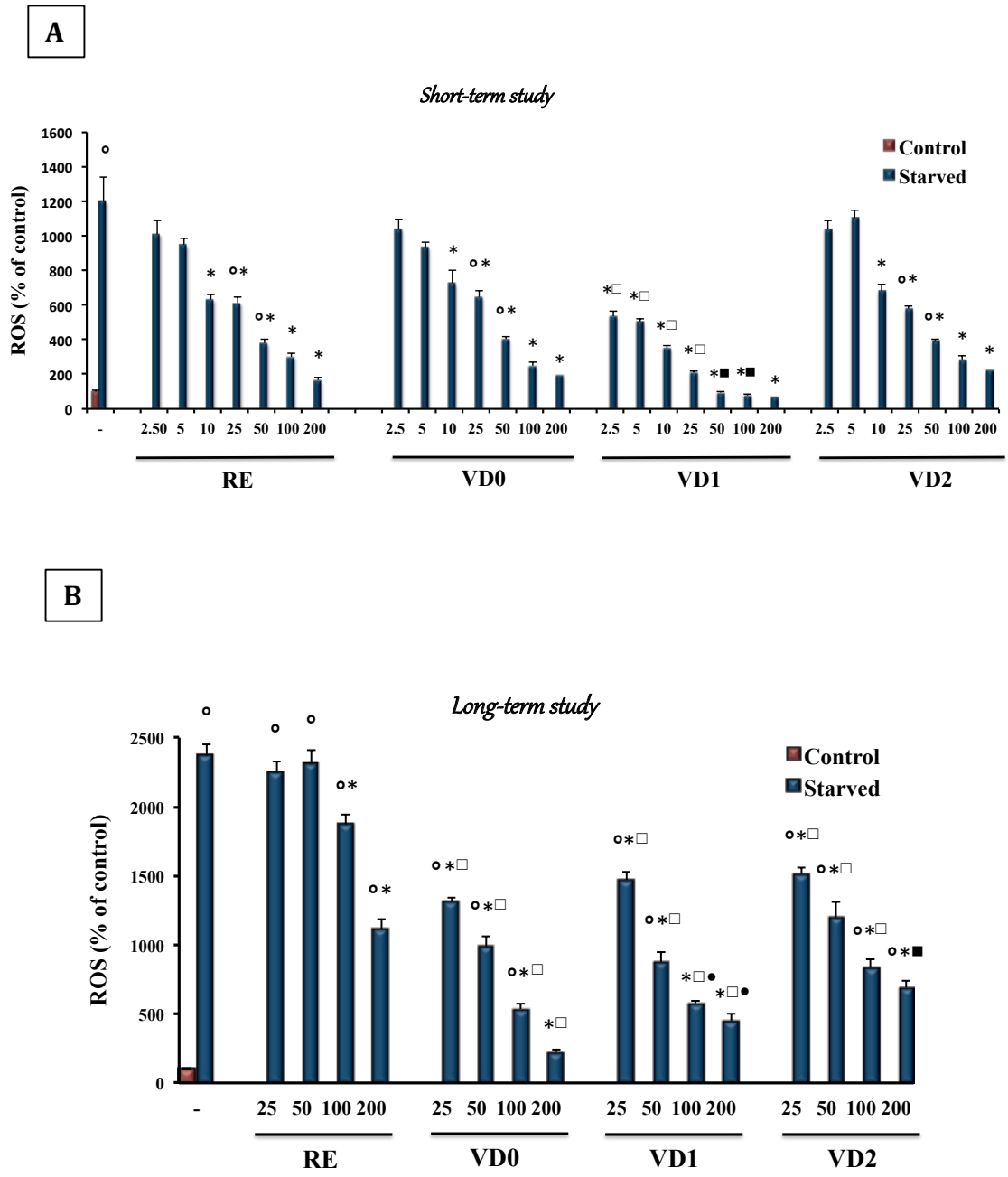

Figure 35. Intracellular ROS production in starved MLO-Y4 cells after short- and long-term treatment with RE and benzoselenophene derivatives. Starved MLO-Y4 cells were treated or not with various concentrations (μM) of RE or VD0, VD1, VD2 derivatives for 3 h (short-term, A) or 24 h (long-term, B) as reported in Materials and Methods. The intracellular ROS production was detected by measuring the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percentage of control (unstarved and untreated cells), are the mean ± SEM of six experiments repeated in triplicate. °P ≤ 0.001 compared to the control MLO-Y4 cells; •P ≤ 0.05 compared to the control MLO-Y4 cells; *P ≤ 0.001 compared to the starved and untreated MLO-Y4 cells; □P ≤ 0.001 compared to the respective RE-treated MLO-Y4 cells; ■P ≤ 0.05 compared to the respective RE-treated MLO-Y4 cells.

In the prevention study, 18Co cells were treated, 1 h before BSO treatment, with concentrations ranging from 10 to 50 μM for RE and from 2.5 to 10 μM for derivatives (Fig. 36A). RE prevented significantly the oxidative stress only at concentration of 50 μM (Fig. 36A). On the contrary, for derivatives this occurred already at the concentration of 5 μM for VD0 and VD2 and with 2.5 μM for VD1. Prevention study was performed also in MLO-Y4 cells treated with concentrations of RE and derivatives ranging from 25 to 200 μM 1 h prior to starvation (Fig. 36B). RE and derivatives, at all concentrations used, prevented significantly ROS production as compared to starved and untreated cells. Moreover, ROS values, detected in the presence of derivatives, were significantly lower than those measured in RE-treated MLO-Y4 cells. Fig. 36B shows that ROS values were not significantly different as compared to those of control in VD0- (50-200 μM), VD1- and VD2- (100-200 μM) treated cells.

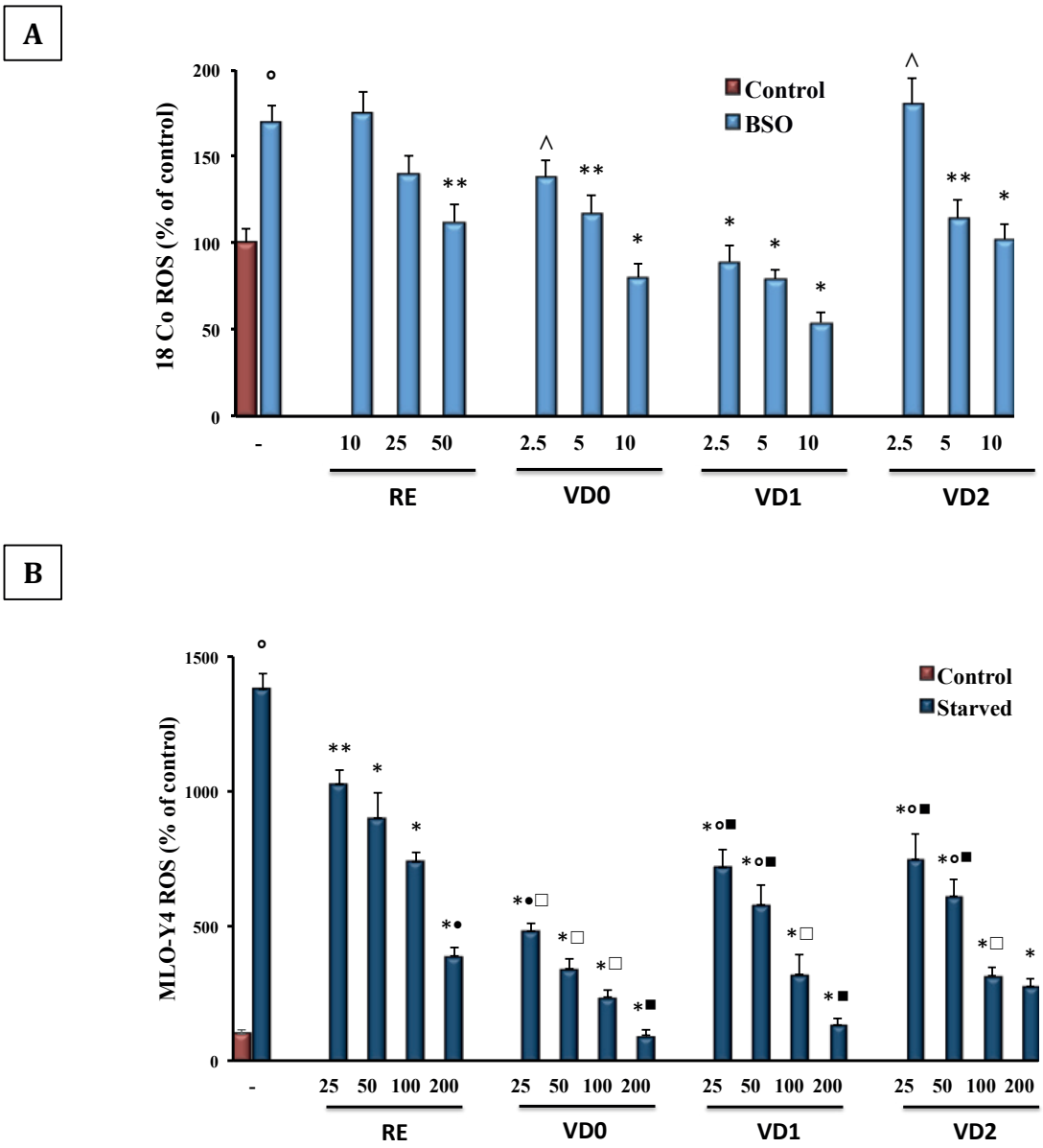

Figure 36. Intracellular ROS production in BSO-treated 18Co cells and in starved MLO-Y4 cells treated with RE and benzoselenophene derivatives in the prevention of oxidative stress. Various concentrations (μM) of RE or VD0, VD1, VD2 were added or not to 18Co (A) and MLO-Y4 (B) cells 1 h before BSO treatment or starvation, respectively, as reported in Materials and Methods. The intracellular ROS production was detected by measuring the intracellular oxidation-sensitive probe H2DCFDA. The values, normalized on total protein content and expressed as percent of control (unstarved and BSO-untreated 18Co or unstarved MLO-Y4), are the mean ± SEM of six experiments repeated in triplicate; °P ≤ 0.001 compared to the control cells; •P ≤ 0.05 compared to the control cells; *P ≤ 0.001 compared to BSO-treated 18Co or starved and untreated MLO-Y4 cells; **P ≤ 0.05 compared to BSO-treated 18Co or starved and untreated MLO-Y4 cells; □P ≤ 0.001 compared to the respective RE-treated cells; ■P ≤ 0.05 compared to the respective RE-treated cells; ^P ≤ 0.05 compared to the respective VD0-or VD1-treated cells.

3. Results III

3.1. Tumor Necrosis Factor-alpha up-regulates ICAM-1 expression and release in intestinal myofibroblasts by redox-dependent and –independent mechanisms.

Fontani F, Domazetovic V, Marcucci T, Vincenzini MT, Iantomasi T

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Paper in appendix

Soluble intercellular adhesion molecule-1 (sICAM-1), an important adhesion molecule that mediates leukocyte-endothelial interaction, has been identified as a marker for the outcome of many diseases, including intestinal bowel diseases, and malignant progression [Witkowska &Borawska, 2004]. In this paper it has been demonstrated that in 18Co the oxidative stress, induced by TNFα or BSO, is responsible of the increased release of sICAM-1 from membranes by metalloproteinases and TACE; effectively literature data report that TACE mediates the release of sICAM-1 [Champagne et al., 1998; Tsakadze et al., 2006; Lawson and Wolf, 2009]. Indeed, the role of TNFα on the increased expression and release of ICAM-1 occurs also by a redox-independent mechanism. For this, the expression and activation of TACE in 18Co cells treated or not with 25 μM BSO and stimulated or not with 1 ng/mL TNFα (TNFα 1) or 10 ng/mL TNFα (TNFα 10) for 24 h, was investigated. TACE expression and activation, detected by Western blot assay, were measured by densitometric analysis of pro-TACE (120 kDa) and mature or active TACE (80 kDa) bands, respectively. These enhanced both in BSO-treated and in TNFα-stimulated cells, as compared to the respective controls (Fig. 37A). Both expression and activation of TACE significantly increased in TNFα 1 + BSO treated cells, when compared to BSO-treated or TNFα 1-stimulated 18Co cells, reaching the values measured in all TNFα 10-stimulated cells.

Phosphorylation of TACE cytoplasmic tail on Thr735 is very important for TACE mobilization to plasma membrane and activation [Scheller et al., 2011]. The use of a specific antibody, which detects endogenous levels of TACE phosphorylation at

Thr735, shows that the trend of this phosphorylation, relative to 80 kDa band, was similar to that of TACE activation in all conditions used (Fig. 37A). However, the proportion of phosphorylated TACE, calculated by ratio between bands of TACE P-Thr735 and those of pro-TACE (120 kDa), was similar in all conditions (Fig. 37B). Moreover, Fig. 38B shows also that mature TACE was all phosphorylated, as indicated by ratio between bands of TACE P-Thr735 and those of TACE at 80 kDa.
NAC and DPI restored TACE expression, activation and phosphorylation to control values in all conditions (Fig. 38A, B).
These data show a strong relationship with ROS levels detected under experimental conditions used (Fig. 39, 40).

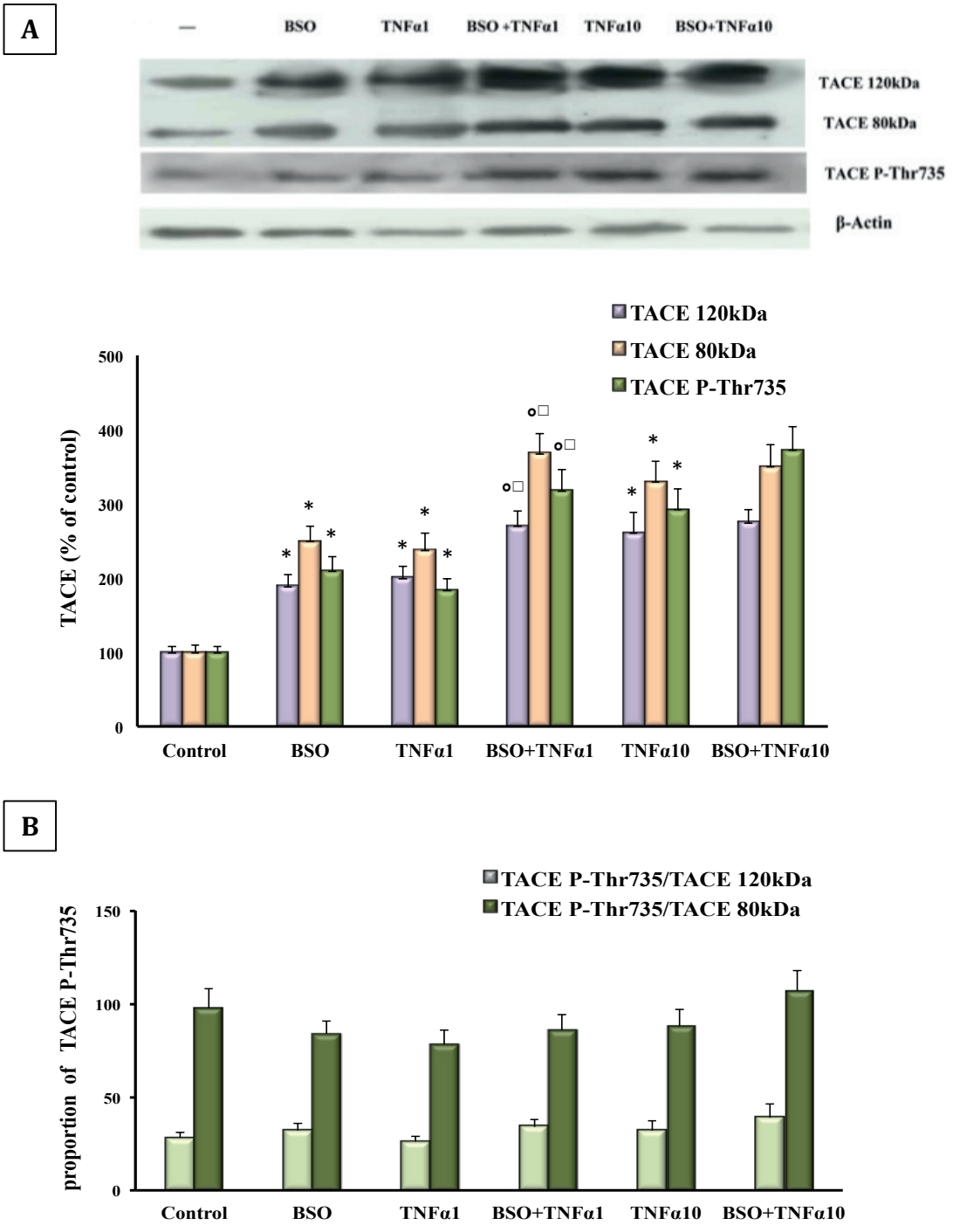

Figure 37. ADAM17 expression and activation in 18Co cells treated or not with BSO and stimulated with TNFα. Starved cells, treated or not with 25 μM BSO as reported in Materials and Methods, were stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). Western blot analysis of cell lysate with anti-TACE or anti-TACE- phosphoThr735 or anti β-actin was performed for the detection of TACE expression and activation. The TACE P-Thr735 was detected at 80 kDa band. A: Blots are representative of four experiments and normalized values with actin band obtained by densitometric analysis of four experiments are reported in the bottom. The values are the mean percentage ± SEM relative to those obtained in the untreated and unstimulated cells (control, 100%). B: The proportion of TACE phosphorylated was calculated both by ratio between bands of TACE P-Thr735 and those of pro-TACE (120 kDa) and by ratio between bands of TACE P-Thr735 and those of mature TACE (80 kDa). *P ≤ 0.005 compared to the control cells; □P ≤ 0.05 compared to the respective TNFα1 stimulated cells °P ≤ 0.05 compared to the respective BSO-treated cells.

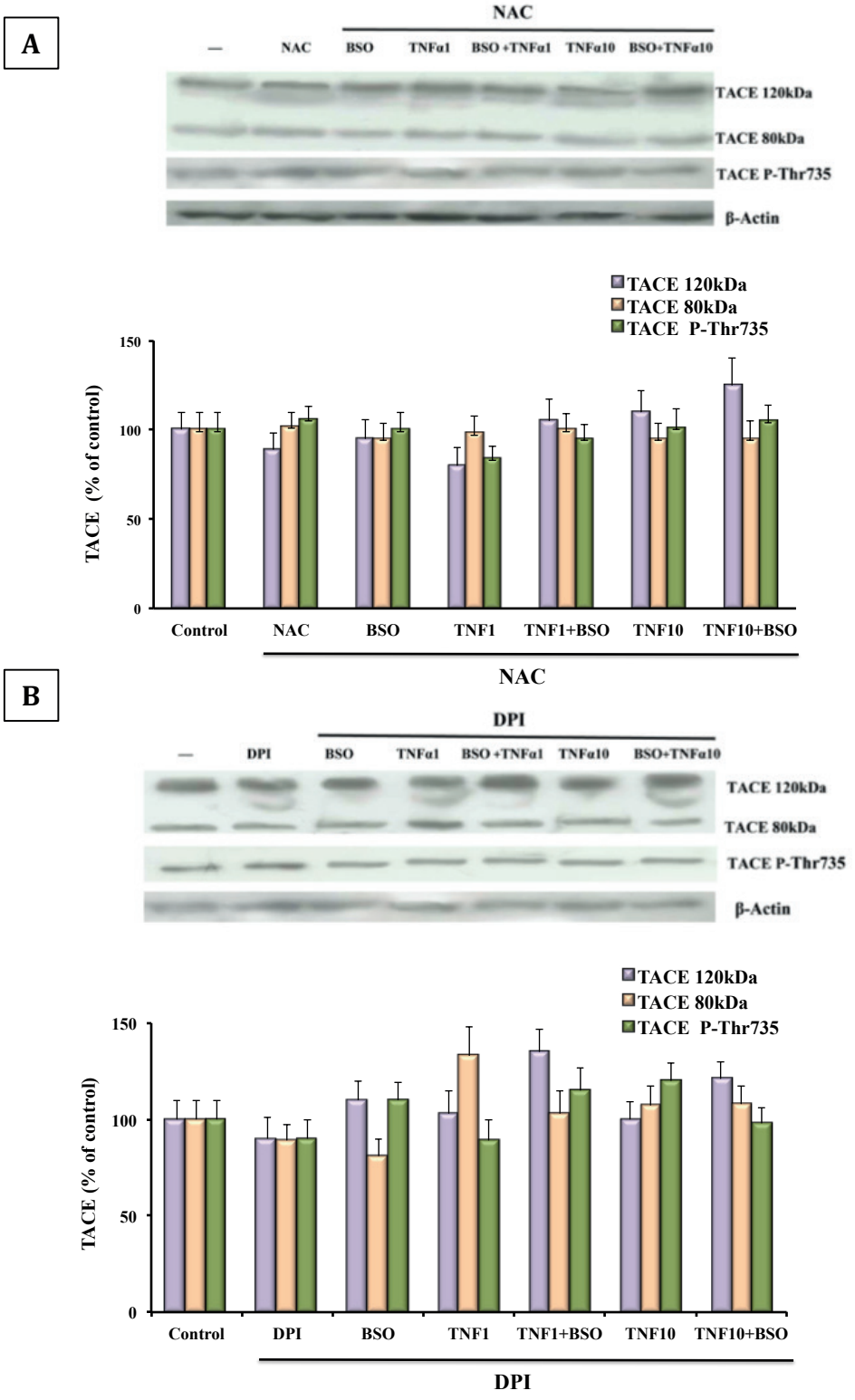

Figure 38. Effect of NAC or DPI on TACE expression and activation in 18Co cells treated or not with BSO and stimulated with TNFα. 20 mM NAC or 10 μM DPI were added to starved cells treated or not with 25 μM BSO and stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10), as reported in Materials and Methods. 0.003% DMSO (vehicle for DPI) was added to DPI untreated cells. Western blot analysis of cell lysate with anti-TACE or anti-TACE-phosphoThr735 or anti β-actin was performed for the detection of TACE expression and activation. The TACE P-Thr735 was detected on 80 kDa band. Blots are representative of four experiments and normalized values with actin band, obtained by densitometric analysis of four experiments. The values are the mean percentage ± SEM relative to those obtained in the untreated and unstimulated cells (control, 100%).

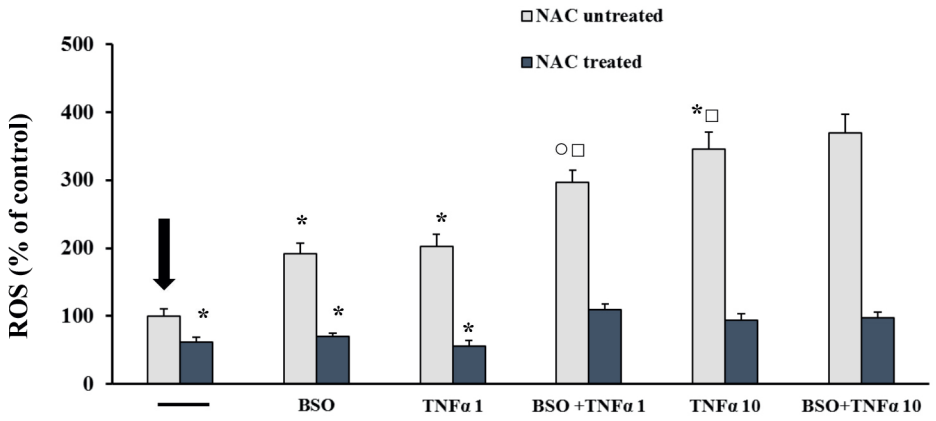

Figure 39. Intracellular ROS production in 18Co cells treated or not with BSO or NAC and stimulated with TNFα. Starved 18Co cells, treated or not with 25 μM BSO or 20 mM NAC as reported in Materials and Methods, were stimulated or not for 24 h with 1ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). The intracellular ROS production was detected by measuring the fluorescence intensity of the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percentage of untreated and unstimulated cells (control, 100%, indicated with the arrow), are the mean ± SEM of six experiments repeated in triplicate; *P ≤ 0.05 compared to the control cells; □P ≤ 0.005 compared to NAC-untreated and TNFα1-stimulated cells; °P ≤ 0.001 compared to NAC-untreated and BSO-treated cells.

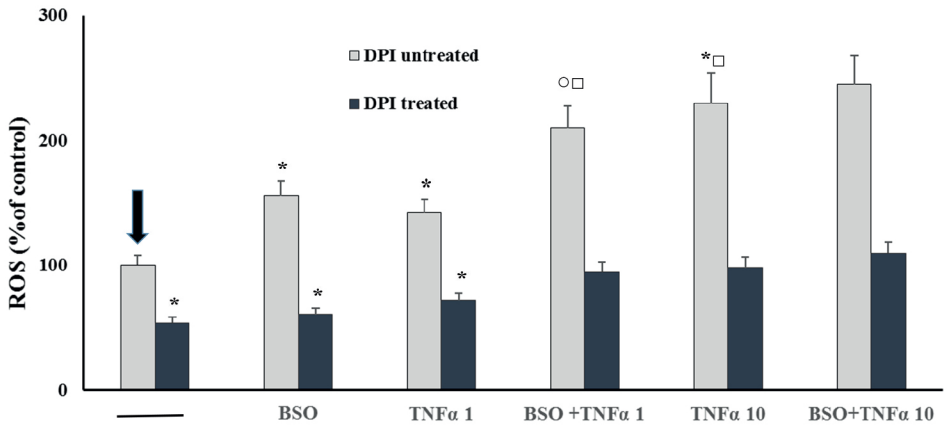

Figure 40. Effect of DPI on intracellular ROS production in 18Co cells treated with BSO and stimulated with TNFα. Starved 18Co cells, treated or not with 25 μM BSO in the presence of 10 μM DPI as reported in Materials and Methods, were stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). 0.003% DMSO (vehicle for DPI) was added to DPI untreated cells. The intracellular ROS production was detected by measuring the fluorescence intensity of the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percentage of untreated and unstimulated cells (control, 100%, indicated with the arrow), are the mean ± SEM of six experiments repeated in triplicate; *P ≤ 0.05 compared to the control cells; □P ≤ 0.005 compared to DPI-untreated and TNFα1-stimulated cells; °P ≤ 0.001 compared to DPI-untreated and BSO-treated cells.

DISCUSSION

1. Discussion I

It has been demonstrated that 17β-E2 quickly reduces apoptosis and the related caspase-3 activation induced by increased oxidative state in starved MLO-Y4 cells, without inhibiting significantly ROS production. The antiapoptotic effect of 17β-E2 in starved MLO-Y4 cells is similar to that obtained after treatment with the antioxidants [Fontani et al., 2015]. In fact, NAC, a direct ROS scavenger, has previously been shown to prevent starvation-induced osteocyte apoptosis due to its remarkable ability of reducing ROS levels at both studied times [Fontani et al., 2015]. Previously it has been demonstrated that osteocyte apoptosis due to starvation is a redox-regulated process. However, although 17β-E2 significantly decreases starvation-induced apoptosis and caspase-3 activation at both the studied times, only after 24 h of starvation it has been registered a weak antioxidant effect of 17β-E2. Some authors demonstrate that the antiapoptotic effect of 17β-E2 is due to a common receptor-independent mechanism which may be related to the antioxidant activity of these molecules [Mann et al., 2007], while others indicate that 17β-E2 may act as inhibitor of NADPH oxidase, a membrane enzyme which increases intracellular oxidative state [Sumi et al., 2003]. Nonetheless, the obtained results do not show a relationship between the antiapoptotic effect of 17β-E2 and its antioxidant activity, which yet seems to be weak, slow and caused by the inhibition of NADPH oxidase activity. Contrarily to this, increased ROS levels and consequent apoptosis at both studied times in starved osteocytes seem to be mainly of mitochondrial origin, and this agrees with the previous findings on the involvement of JNK activation in the apoptosis of MLO-Y4 cells [Fontani et al., 2015]. Indeed, the ability of JNK in mediating oxidative stress-induced apoptosis depends prevalently on its interactions with mitochondrial pathways [Davis, 2000], and this may occur in osteocytes through increased permeability of the outer mitochondrial membrane with the release of cytochrome c from the mitochondria into the cytoplasm. Cytochrome c promotes activation of latent caspases (caspase-2, -8, -9, and -10) which subsequently

activate the effector caspases (caspase-3, -6 and -7) responsible for programmed cell death [Jilka et al., 2013]. Effectively, it seems difficult for 17β-E2 to have a direct antioxidant effect as shown by Mann et al. in whose study on MLO-Y4 cells the apoptosis was induced by extracellular H_2O_2 (0.3 mM) and prevented by 10 nM 17β-E2. It is also improbable for estrogens to have a direct antioxidant effect *in vivo* given that their circulating levels are lower than those of the antioxidants needed to reduce the oxidative stress [Borrás et al., 2005; Bellani et al., 2013]. Indeed, studies *in vitro* and *in vivo* demonstrate that 17β-E2 antioxidant action is related its ability to bind to estrogen receptors and to up-regulate the expression of antioxidant enzymes such as SOD and GPX [Borrás et al., 2005; Almeida et al. 2007, 2010; Bellani et al., 2013, Doshi & Agarwal, 2013]. However, this mechanism may require longer time respect to direct antioxidant action, and this study suggests that the early antiapoptotic effect of 17β-E2 occurs in the presence of an oxidative state.

As previously reported by Fontani et al. 2015, oxidative stress activates MAP kinases JNK and ERK1/2 in starvation-induced osteocyte apoptosis, but only JNK activation is involved in oxidative stress-induced apoptosis in MLO-Y4, and the antioxidants, including NAC, are able to inhibit both JNK and ERK1/2 activity by decreasing ROS levels. Similarly, to antioxidants, 17β-E2 inhibits JNK activation in starved MLO-Y4 cells at both studied times, and this can be related to the antiapoptotic effect of 17β-E2 but not to the decreased ROS levels. This indicates that 17β-E2-antiapoptotic effect occurs through a process which is not related to oxidative status but to a redox-independent JNK inactivation. Moreover, 17β-E2 does not affect ERK1/2 activation due to its inability to mitigate the oxidative stress, indicating that ERK1/2 activation is not involved in the 17β-E2 protective effect against osteocyte apoptosis, similarly to that observed by Mann et al. in osteocyte apoptosis induced by H_2O_2 treatment. No data are reported in literature regarding the protective effect of 17β-E2 on oxidative stress-induced apoptosis in osteocytes by down-regulation of JNK activity. For this purpose, the role of specific dephosphorylating agents such as MKP-1, a member of the MKP family, that predominately dephosphorylates JNK [Carlson et al., 2009; Takeuchi et al., 2009], was studied in estrogen-induced JNK inactivation. Indeed, 17β-E2 can induce genomic expression of MKP-1 in various cells including bone cells by acting

through its receptors [Takeuchi et al., 2009]. Moreover, it has been shown that MKP-1-modulated JNK activity is critical for induction of apoptosis due to the inhibition of epidermal growth factor receptor tyrosine kinase in lung cancer cells [Takeuchi et al., 2009]. Effectively, MKP-1 is expressed in MLO-Y4 but, under the studied experimental conditions, 17β-E2 as well as the increased oxidative state do not induce an overexpression of this phosphatase, thus excluding MKP-1 possible role on JNK inactivation by 17β-E2.

The role of GSTP1-1, a detoxifying enzyme also involved in cell death and/or survival mechanisms, was also investigated and found to be expressed in osteocytes. Furthermore, for the first time, it has been shown that GSTP1-1 is related to the antiapoptotic effect of 17β-E2 mediated by JNK inactivation in the presence of oxidative state. In particular, it is well known that GSTP1-1 regulates JNK signalling pathway through the formation of GSTP1-1/c-Jun/JNK complex. This association occurs with GSTP1-1 monomeric form and selectively inhibits the phosphorylation and activation of JNK [Adler et al., 1999; Bernardini et al., 2000; Bartolini & Galli, 2016; Laborde et al., 2010; Okamura et al., 2015]. Under normal physiological conditions, there is a reversible equilibrium among GSTP1-1 monomeric and dimeric and/or polymeric forms, that shifts versus polymeric forms in the presence of oxidative state due to the formation of disulphide bonds [Davis, 2000; Okamura et al., 2015; Bartolini & Galli, 2016], thus inducing GSTP1-1/JNK dissociation and apoptosis activation [Adler et al., 1999; Bernardini et al., 2000; Laborde et al., 2010; Okamura et al., 2015]. Immunoprecipitation and immunoblot experiments demonstrate that JNK is bound to GSTP1-1 in MLO-4Y cells. In agreement with literature data, immunoprecipitation and immunoblot experiments demonstrate that in MLO-Y4 cells JNK is associated to GSTP1-1 and under conditions of oxidative stress both the levels of JNK-associated and GSTP1-1 monomeric form decrease in starved cells. This is inversely related to JNK activation that increases only in the presence of high levels of ROS, suggesting JNK/GSTP1-1 dissociation. This redox regulation of GSTP1-1/JNK association is confirmed by findings obtained with NAC treatment of MLO-Y4 cells. In fact, NAC preserves GSTP1-1/JNK association, preventing complex dissociation and, therefore, JNK activation maintaining high levels of GSTP1-1 monomeric form by decreasing ROS levels and reducing disulphide bonds present in GSTP1-1

oligomeric forms. The data show that 17β-E2 is also able to maintain high levels of both GSTP1-1 monomeric and JNK-associated form. This indicates that 17β-E2 may preserve GSTP1-1/JNK complex dissociation with the consequent JNK inactivation even in the presence of oxidative stress. In particular, the effect of 17β-E2 can be related to the overexpression of GSTP1-1, according to literature data showing that the overexpression of GSTP1-1 can affect the balance between various forms of GSTP1-1 and its possible association with JNK [Adler et al., 1999; Bernardini et al., 2000; Laborde et al., 2010; Bartolini et al., 2015; Okamura et al., 2015]. Indeed, the increased GSTP1-1 expression can shift the equilibrium towards the formation of the GSTP1-1/JNK complex, and this counteracts the action of ROS that induce complex dissociation and JNK activation. This mechanism has been demonstrated also in the tumour cell resistance to apoptosis [Adler et al., 1999; Bernardini et al., 2000; Okamura et al., 2015], and in agreement with our data, Bartolini et al. 2015 reported that GSTP1-1 overexpression inhibits stress-induced JNK activation, whereas it does not affect ERK1/2 activation.

Direct involvement of GSTP1-1 in the protective effect of 17β-E2 and NAC on oxidative stress-induced apoptosis and on modulation of JNK activation, has been shown by GSTP1-1 down-regulation. In particular, GSTP1-1 seems important in mediating the early antiapoptotic effect of 17β-E2 through the inhibition of JNK in the presence of oxidative stress. In 17β-E2-treated cells, down-regulation of GSTP1-1 reverts similarly to starved cell values both apoptosis and JNK activation. In NAC-treated cells, down-regulation of GSTP1-1 also increased both apoptosis and JNK activation, however, these do not return to starved cell values, thus indicating that stress-induced JNK activation followed by apoptosis, may also in part depend on other GSTP1-1-independent redox-regulated factors.

Previously, we demonstrated that when there is an oxidative state in MLO-Y4 the expression of factors, such as RANKL, OPG and sclerostin involved in bone remodelling, are related to apoptosis as well as to JNK and ERK1/2 activity [Fontani et al., 2015]. Indeed, RANKL, sclerostin and OPG are expressed by osteocytes under various conditions, including bone pathological alterations [Henriksen et al., 2009; Bonewald, 2011; Mulcahy et al., 2011; Khosla et al., 2012; Doshi & Agarwal, 2013; Manolagas et al., 2013; Park, 2013; Fujita et al., 2014]. RANKL increases osteoclast differentiation and bone resorption, whereas OPG

competes with RANKL for its receptor thus inhibiting osteoclastogenesis. Moreover, it has been shown that RANKL/OPG ratio is indicative of osteoclastogenic activity in various bone remodelling diseases and it maintains bone resorption and bone formation processes in equilibrium [Henriksen et al., 2009; Bonewald, 2011; Mulcahy et al., 2011; Khosla et al., 2012; Manolagas et al., 2013; Fujita et al., 2014]. Sclerostin, mainly produced by mature osteocytes, is a negative regulator of osteoblast activity and OPG production by inhibiting the Wnt/β-catenin signalling pathway responsible for OPG production [Krum et al., 2008; Henriksen et al., 2009; Bonewald, 2011; Khosla et al., 2012; Manolagas et al., 2013; Fujita et al., 2014]. 17β-E2 is able to prevent the increased expression and release of RANKL and the increased expression of sclerostin, however, without reverting their levels to those of control, differently to antioxidants that in MLO-Y4 cells inhibit both JNK and ERK1/2 activity [Fontani et al., 2015]. Similarly, to antioxidants, 17β-E2 is also able to prevent in part the remarkable decrease in OPG levels observed in starved osteocytes, as only JNK activity is involved in the down-regulation of OPG expression [Fontani et al., 2015]. However, since both 17β-E2 and antioxidants almost totally inhibit JNK activity, their inability to restore normal OPG levels can be due to the involvement of other JNK- and/or oxidative stress-independent factors and likely related to starvation. All these data show that the effect of 17β-E2 on expression of the studied bone remodelling factors is mainly mediated by JNK inhibition. 17β-E2 remarkably decreases RANKL/OPG ratio after 24 h from starvation because only at this time 17β-E2 significantly increases OPG levels. However, differently from the antioxidants, 17β-E2 does not revert the ratio values to those of the control [Fontani et al., 2015] probably because of the inability of estrogen to quickly eliminate ROS and to inhibit ERK1/2 activation that affects RANKL expression.

All these data show that the quick effect of 17β-E2 on the expression of RANKL, sclerostin and OPG in the presence of oxidative state depends mainly on GSTP1-1 expression that affects JNK activity. In particular, the effect of 17β-E2 on the up-regulation of OPG may be related to the down-regulation of sclerostin expression, and this event may be related to 17β-E2 modulation of JNK activity by up-regulation of GSTP1-1. This agrees with a potential crosstalk between the Wnt/β-catenin signalling pathway and estrogen signalling [Bonewald, 2011, 2008;

Henriksen et al., 2009]. Thereafter, the regulation of GSTP1-1/JNK association may be related to 17β-E2 signalling and RANKL/OPG ratio levels. In osteocytes, this is important as it supports the efficacy of estrogen treatment in postmenopausal women by decreasing sclerostin and RANKL levels, which result enhanced in serum during estrogen deficiency [Mödder et al., 2011; Khosla et al., 2012; Manolagas et al., 2013]. Indeed, high levels of both RANKL and sclerostin are implicated in the increased bone resorption associated with estrogen deficiency and microdamage [Almeida et al., 2007; Rochefort et al., 2010; Mulcahy et al., 2011; Jilka et al., 2013; Sapir-Koren & Livshits, 2013].

Conclusion:

A novel and important role of GSTP1-1 in the effect of 17β-E2 at molecular level in osteocyte activity, has been identified. 17β-E2 prevents oxidative stress-induced osteocyte apoptosis in MLO-Y4 cells by inhibition of JNK activation, through up-regulation of GSTP1-1 expression thus contributing to the maintenance of JNK/GSTP1-1 association that inhibits JNK activity (Fig. 39). This association is regulated by intracellular redox changes, as in the case of NAC, and/or by GSTP1-1 overexpression, as in the case of 17β-E2. Moreover, 17β-E2 by JNK inhibition decreases RANKL/OPG ratio and sclerostin levels, important factors related to osteogenesis and bone remodelling. Moreover, these data identify a possible role of GSTP1-1 and JNK activity in bone repair mechanisms in pathologies related to oxidative stress.

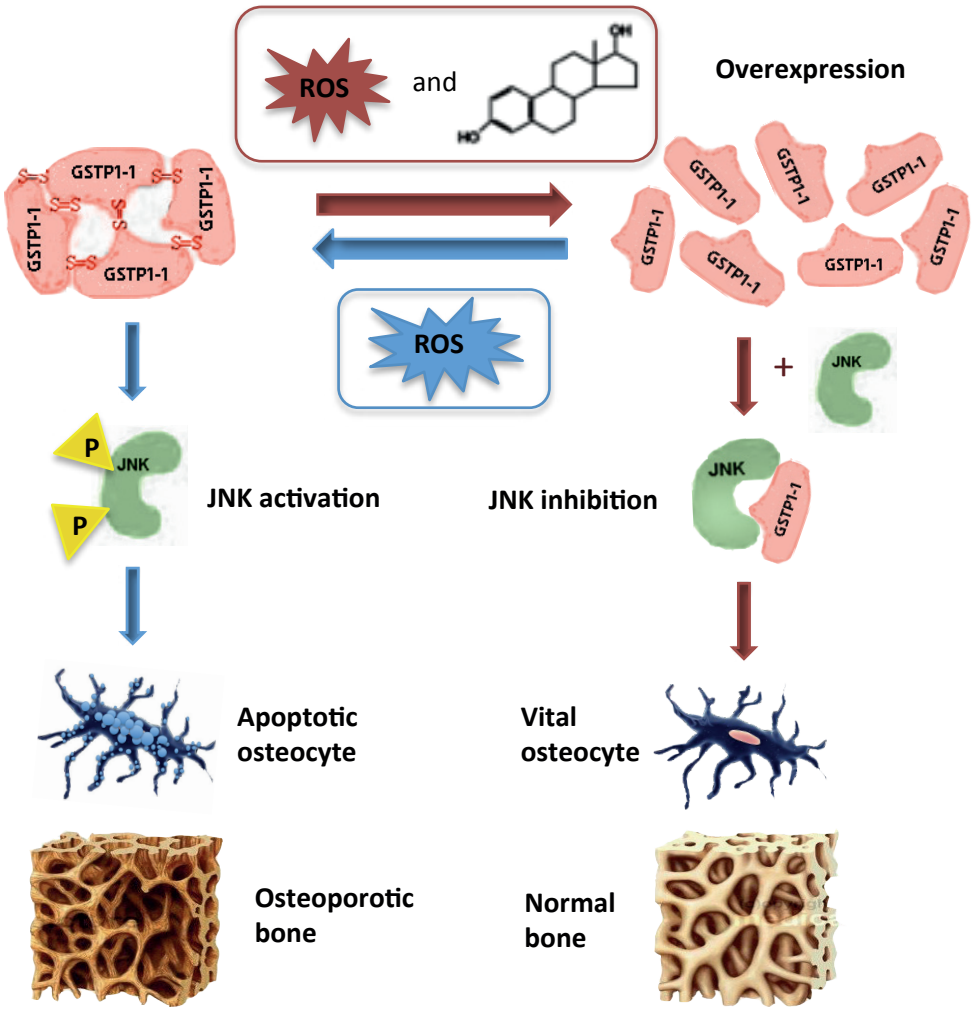

Figure 39. Summary of the effects of oxidative stress and 17β-E2 on JNK/GSTP1-1 association on osteocyte activity and bone remodelling.

The consumption of blueberries is considered an important contribution to the diet, especially if they are consumed fresh during summertime between June and August. This is due to the abundance of various classes of phenolic compounds, that make this fruit rich in anti-inflammatory, anti-hypertensive, antimicrobial and anticancer properties. Among the bioactive substances present in blueberries, flavonoids, and specifically anthocyanins, have been proven to dispose a strong antioxidant capacity [Wang et al., 1997]. Nowadays, blueberries are commercialised in different ways, mainly as fresh or frozen products. Most

studies to determine the composition of bioactive compounds as antioxidants derive from dried extracts. However, freezing, drying and treatment with solvents (i.e. for the production of dry extracts) are possible methods to preserve blueberries but the severity of those processes might destroy in part anthocyanins or their antioxidant effects [Lohachoompol et al., 2004]. Moreover, the anthocyanins, polyphenols widely present in *Vaccinium mirtillus*, seem to be more stable over time in juice at acidic pH rather than in a dry extract. So blueberry juice as a dietary supplement seems to be preferable to dried extracts put into tablets, the juices are the fastest preparations available at home and for the most economical manufacturers. Our data show that there are no differences between the two types of preparation in antioxidant and antiapoptotic properties in starved osteocytes. In particular, the results show that both fresh juice, obtained from blueberries native in Italian Apennines where is prevalently present *Vaccinium myrtillus*, and blueberry dry extract reduce the apoptosis induced by oxidative stress in starved MLO-Y4 osteocytes in the same manner as GTE and HE containing equal amount of TSP. However, blueberry antiapoptotic effect is not closely related to its antioxidant effect in MLO-Y4 cells, differently to that observed in GTE- and HE-treated MLO-Y4 cells. This suggests that antiapoptotic effect of blueberries can be also due to other factors, perhaps not strictly redox regulated, which remain to be elucidated in further studies. Moreover, the ability of blueberry preparations to prevent the increase of osteoclastogenic factors, such as RANKL and sclerostin, in the presence of oxidative stress is similar to those of GTE and HE known for their beneficial effect in counteracting the loss of bone mass.

For the first time it has been demonstrated a possible role of blueberries in osteocyte activity involved in regulation of the bone remodelling process and this may be due to various biologically active phytochemicals contained in preparations of these plants, which possess beneficial effects for maintenance of osteogenic activity of osteocytes in the presence of oxidative stress.

Conclusion:

These data indicate that blueberries may influence bone turnover through prevention of oxidative-stress induced apoptosis and expression of osteoclastogenic factors in osteocytes. This effect is mainly due to its antioxidant component, although the involvement of other components in modulating these

effects cannot be excluded. Therefore, dietary supplementation with blueberries may be useful in the development of nutraceuticals or as functional food ingredient useful for inhibiting bone resorption associated with bone diseases related to oxidative stress, such as osteoporosis.

2. Discussion II

The antioxidant effect of RE and its synthesized benzoselenophene derivatives (VD0, VD1 and VD2) was detected in both MLO-Y4 and 18Co cells. The oxidative stress condition was experimentally induced differently in both cells types; in particular, in MLO-Y4 by starvation, while in 18Co by treatment with BSO, a compound normally used to induce GSH deficiency [Mårtensson et al., 1991]. The starvation itself does not induce oxidative stress in 18Co cells differently to that occurs in MLO-Y4. These two conditions are considered valid *in vitro* models for creating the conditions of oxidative stress present in pathologies such as bone inflammatory disorders or IBD [Catarzi et al., 2011; Fontani et al., 2014, 2015; Domazetovic et al., 2017].

ROS production in starved MLO-Y4 cells is significantly higher than that detected starved BSO-treated 18Co, and the concentrations of RE and its benzoselenophene derivatives used do not have cytotoxic effects in both cell types as confirmed by their antioxidant effect.

Literature data show that a variety of resveratrol analogues have been synthesized and their effect on oxidative stress is structure- and cell type-related [Bhuyan et al., 2012; Selma et al., 2012; Gobec et al., 2015]. In fact, hydroxylated resveratrol analogues enhance ROS production in cancer cells by reducing proliferation and increasing apoptosis [Kucinska et al., 2014], while they have remarkably higher antioxidant ability than RE in rat liver microsomes [Cai et al., 2003]. Tricyclic derivatives of RE containing hydroxy and/or methoxy substituents in determined positions on the phenolic rings, show the same or minor antioxidant effect than RE itself in a human leukemic monocyte-like cell line, THP-1 [Gobec et al., 2015].

In the short-term study, VD0 and VD1, differently to VD2, restore ROS levels to control values at a concentration that is half of that required for RE in 18Co. On the contrary, in MLO-Y4 cells only VD1 restores ROS levels to control values. The data

from the short-term study indicate that VD1 possesses higher antioxidant power than RE and other derivatives.

In the long-term study, derivatives are more efficient than RE in reducing oxidative stress in ML0-Y4 cells, however restoration of the redox state occurs only with 200 μM VD0, while in 18Co cells all derivatives restore ROS production to control values at concentrations 20 times lower than that of RE.

Also, in the prevention of oxidative stress benzoselenophene derivatives are more effective than RE in both MLO-Y4 and 18Co cells, in fact, their preventive effect is complete at concentrations lower than those of RE.

The data observed show that RE benzoselenophene derivatives reduce oxidative stress in MLO-Y4 and 18Co cells much more than RE itself, confirming what observed in a non-biological *in vitro* system [Tanini et al., 2015]. Indeed, benzoselenophene derivatives are able to restore the physiological redox state in MLO-Y4 and in 18Co, although this effect is achieved at different concentrations and can be due to degree of oxidative stress, cell type and structural characteristics of compounds.

Considering the high antioxidant capacity of RE benzoselenophene derivatives both in down-regulation and/or prevention of oxidative stress, the potential use of these compounds may be useful in reducing the inflammatory state in IBD or excessive bone resorption in bone pathological disorders.

Moreover, being RE quickly absorbed and metabolized [Vang et al., 2011], and therefore has difficulty in reaching the distal part of gut, the possible use of derivatives of RE, such as glucosylacyl and glucosyl, has already been proposed in IBD treatment. In fact, these compounds block the adherence of pathogens to human colonic cells more efficiently than RE and prevent intestinal inflammation *in vivo* [Larossa et al., 2010; Selma et al., 2012]. Moreover, anti-osteoporotic role of RE has been demonstrated *in vivo* the [Tou et al., 2015], and RE-synthesized oligomers display anti-osteoporotic activity in the ovariectomized female rats [Hao et al., 2015].

Conclusion:

Benzoselenophene derivatives of RE are more effective in restoring physiological redox state as compared to RE. They act at lower concentrations than RE that did not reduce efficiently oxidative stress after the long-term treatment. In

particular, VD1 and all derivatives have major antioxidant effect in MLO-Y4 after short-term and long-term treatments, respectively. In 18Co cells the highest antioxidant effect has been observed with VD1 during the prevention study. It has been speculated the use of benzoselenophene derivatives as an alternative therapy and/or therapeutic support in intestinal inflammatory disease and osteoporosis. In fact, oxidative stress is responsible of an increased production of inflammatory mediators by intestinal myofibroblasts as well as of enhanced osteoclastogenic factors and bone loss due to osteocytes.

3. Discussion III

For the first time, in 18Co cells an up-regulation of the expression and activation of TACE, related to intracellular oxidative stress, has been demonstrated. Indeed, alterations of the cellular redox state may regulate both activation and expression of TACE [Champagne et al., 1998; Tsakadze et al., 2006; Lawson and Wolf, 2009; Scheller et al., 2011], but in literature no data are reported on the expression, activation and regulation of this enzyme in relationship to the intracellular oxidative state in intestinal myofibroblasts. The data obtained indicate that TACE expression and activation correlate with ROS levels in cells treated with BSO and/or stimulated with TNFα. Furthermore, this was confirmed by the antioxidant action of NAC treatment that restores TACE levels to control values in all conditions. Also, DPI treatment induces the same effect observed with NAC thus indicating the involvement of NADPH oxidase in the redox regulation of TACE.

Wang et al. 2009 demonstrated that exogenous H_2O_2 activates TACE, involving the cysteinyl sulfhydryl groups of the extracellular domain, and the increase of TACE cell surface levels is due to p38-mediated TACE phosphorylation [Brill et al., 2009]. In fact, the activation of p38 MAPK leads to specific phosphorylation of Thr735 involved in the TACE translocation to membrane and its subsequent activation. In the experimental conditions used in 18Co cells, Thr735 phosphorylation trend relative to 80 kDa band is in agreement with TACE activation and it is related to ROS levels.

The increased phosphorylation of TACE correlates also with the up-regulation of its expression. In fact, the absolute values of phosphorylated TACE change, whereas the proportion of phosphorylated TACE respect to its expression levels is similar in all experimental conditions. The involvement of p38 MAPK on TACE phosphorylation could be plausible considering that a redox-regulated activation of this MAPK occurs in 18Co cells stimulated or not with TNFα and in intestinal myofibroblasts isolated from colon mucosa of CD patients [Catarzi et al., 2011].
Therefore, this redox regulation of TACE expression and activation, detected in intestinal myofibroblasts, may be involved in the pro-inflammatory activity of TNFα in inflamed gut. In fact, myofibroblasts produce TNFα in intestinal inflammatory state and show high expression of transmembrane TNFα in CD patients [Di Sabatino et al., 2007]. Moreover, TACE is required in cell surface proteolytic processing of TNFα that after cleavage assumes its inflammatory properties [Scheller et al., 2011].

Conclusion:

The obtained results demonstrate that in myofibroblasts the expression and activation of TACE may be regulated by intracellular redox state. In fact, oxidative stress increases TACE expression and activation, whereas NAC down-regulates these events by reducing ROS levels. Consequently, the release of sICAM-1 and pro-inflammatory cytokines can be also regulated by intracellular redox state through TACE levels and activity. For this reason, antioxidants may have beneficial role in reducing the activation of inflammatory cascade and preventing the progression of chronic inflammatory disease.

General Conclusion

Overall, the results of this study could be useful to identify biological targets for novel pharmacological approaches for bone and chronic inflammatory bowel diseases related to oxidative stress. Moreover, they confirm that natural and/or synthetic antioxidant compounds can have beneficial effects as both dietary and pharmacological supplements for their preventive, synergic and low side effect profile.

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Estrogen inhibits starvation-induced apoptosis in osteocytes by a redox-independent process involving association of JNK and glutathione S-transferase P1-1

Vladana Domazetovic[1]*, Filippo Fontani[1]*, Gemma Marcucci[2], Teresa Iantomasi[1], Maria Luisa Brandi[2] and Maria Teresa Vincenzini[1]

1 Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", (Biochemistry section) University of Florence, Italy
2 Department of Surgery and Translational Medicine (Endocrinology Section), University of Florence, Italy

Keywords
estrogen; GSTP1-1 expression; JNK activity; osteocyte apoptosis; RANKL/OPG ratio

Correspondence
M. T. Vincenzini, Department of Biomedical, Experimental and Clinical Sciences, University of Florence, Viale Morgagni 50, 50134 Florence, Italy
Fax: +39055 7830303
Tel: +39055 2751238
E-mail: vincenzini@unifi.it

*These authors contributed equally to this work.

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Estrogen deficiency causes bone loss as a result of microdamage, oxidative stress, and osteocyte apoptosis. A relationship between oxidative stress-induced apoptosis, c-Jun N-terminal kinase (JNK) activation, and expression of factors involved in bone remodeling has been demonstrated in osteocytes. However, the molecular regulation of these events in osteocytes treated with 17β-estradiol (17β-E2) remains unexplored. The MLO-Y4 murine osteocyte-like cell line was used as a model to study starvation-induced apoptosis and ROS production during 17β-E2 treatment. Expression of glutathione S-transferase P1-1 (GSTP1-1), receptor activator kB ligand (RANKL), osteoprotegerin (OPG), sclerostin, and kinases activation were measured by western blot. In addition, the GSTP1-1/JNK association was assessed by immunoprecipitation, and GSTP1-1 involvement in the osteocyte response to 17β-E2 was detected by specific siRNA transfection. 17β-E2 prevents starvation-induced apoptosis (DNA fragmentation and caspase activation), the increase in sclerostin expression and the RANKL/OPG ratio, which are all related to JNK activation due to oxidative stress in osteocytes. This occurs through GSTP1-1 overexpression, which can inhibit JNK activation by formation of a GSTP1-1/JNK complex. No early antioxidant action of 17β-E2 has been found but the estrogen effect is similar to N-acetylcysteine which, by increasing the intracellular redox state, maintains JNK bound to GSTP1-1. Thus, the antiapoptotic and osteogenic effect of 17β-E2 in MLO-Y4 occurs by a redox-independent process involving GSTP1-1/JNK association. This study clarifies at molecular level the effect of 17β-E2 on osteocyte activity and identifies a possible role of GSTP1-1 and JNK activity in bone remodeling and repair mechanisms.

Estrogens influence the size and shape of the skeleton during growth and contribute to bone homeostasis during adulthood. The decline in estrogen levels, associated with menopause, causes bone loss in women related to a high rate of bone remodeling [1,2] which results from the close balance between osteoblast and

Abbreviations
17β-E2, 17β-Estradiol; AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride; DPI, diphenyleneiodonium; ERK1/2, extracellular signal-regulated kinase; GSTP1-1, glutathione S-transferase P1-1; JNK, c-Jun N-terminal kinase; Mito TEMPO, 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl-triphenylphosphonium chloride monohydrate; MKP-1, protein kinase phosphatase-1; NAC, N-acetylcysteine; OPG, osteoprotegerin; RANKL, receptor activator kB ligand; ROS, reactive oxygen species; scr siRNA, scrambled siRNA.

osteoclast activity, leading to either bone formation or bone resorption, respectively. Recently, new data support the central regulatory role of osteocytes in the maintenance of this balance and thus in viability and functionality of bone [3,4]. Estrogen deficiency alters osteoblast mineralization and osteoclast resorption activity, leading to increased bone resorption and osteoporotic cascade [1,2,5]. In fact, estrogens, through their receptors, increase bone mineralization [1,2,6,7], whereas estrogen deficiency causes an increase in osteoblast and osteocyte apoptosis in both the trabecular and cortical compartment [1]. This causes a deteriorated microarchitecture and reduced mechanical strength of bone as well as increased fracture risk [1,8]. The marked reduction in estrogen induces oxidative stress related to menopause and bone loss [9–12]. In fact, it has been demonstrated that, in addition to aging, ovariectomy induces oxidative stress in rat femurs along with decreased activity of antioxidant systems [12]. Moreover, the protective effects of estrogens on bone result from their ability to attenuate oxidative stress [13] by activating antioxidant gene expression [9–11,13] or by a direct free radical scavenging activity [14]. However, other data indicate that estrogen therapy has no significant effect on oxidative stress levels [11,15].

Many studies, performed on osteoblasts and osteoclasts, have linked reactive oxygen species (ROS) and antioxidants to bone metabolism and bone remodeling [13,16–18]. In osteocytes, high levels of ROS induce increased apoptosis [14,19–21], and this is also related to loss of estrogens and a decrease in bone mineral density [12,22,23], as occurs in aging and chronic glucocorticoid treatment [12,19,22,24–27]. Since osteocytes are mechanosensitive cells [3,4], increased apoptosis may impair the ability of bone to adaptively respond to mechanical loading and to repair microdamage due to physiological or pathological events such as aging, osteoporosis, or osteoarthritis, also related to oxidative stress [3,19,22–26]. It has been demonstrated that the modulation of mechanical loading and ovariectomy induce an increase in osteocyte apoptosis and lead to microdamage [19,22,23] with subsequent osteoclastic invasion to the damaged site and activation of the remodeling process [28–31]. Estrogen treatment prevents osteoblast and osteocyte apoptosis [1,2,5,13,14,23], although molecular mechanisms and biochemical signals, by which estrogens protect osteocytes from the apoptosis due to microdamage and/or oxidative stress, have not been fully elucidated. Similarly, to our knowledge in osteocytes, no data are reported on the molecular processes by which estrogens modulate the expression of cytokines involved in bone remodeling in the presence of oxidative stress-induced apoptosis.

The aim of this study was to investigate the molecular processes by which 17β-Estradiol (17β-E2) prevents osteocyte apoptosis and the abnormal expression and release of factors related to increased oxidative state and bone remodeling. The effect of 17β-E2 on ROS production and the activation of redox-regulated kinases, such as extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK), were also studied. Indeed, in osteocytes, these kinases are involved in oxidative stress-induced apoptosis and expression of receptor activator kB ligand (RANKL), osteoprotegerin (OPG), and sclerostin [21]. To clarify the molecular action of estrogen on these events, the role of mitogen-activated protein kinase phosphatase-1 (MKP-1) and glutathione S-transferase P1-1 (GSTP1-1), both involved in the regulation of JNK activity, were investigated [32–35]. This study was performed in MLO-Y4, a murine osteocyte-like cell line, that constitutes an *in vitro* model for studying osteocyte viability and apoptosis in response to microdamage and bone diseases [31,36–38]. Apoptosis due to oxidative stress was induced in MLO-Y4 by serum starvation [21]. This method causes apoptosis not due to proinflammatory and proapoptotic factors and mimics *in vitro* a metabolic condition of oxidative stress that may be similar to what occurs *in vivo* in the bone environment after microdamage [19,20,31,37–39]. In fact, apoptosis due to microdamage may be related to a disruption of blood and fluid flow with consequent lack of various endocrine factors, including estrogens [31,40,41]. Thus, it is possible to investigate "*in vitro*" the regulatory role of estrogens in osteocyte apoptosis in bone remodeling.

Materials and methods

MLO-4Y culture and treatment

MLO-4Y (a gift from L. Bonewald, University of Missouri-Kansas City) were cultured at 37 °C in a 5% CO_2 humidified atmosphere in alpha-MEM medium supplemented with 5% calf serum (GE Healthcare HyClone, Little Chalfont, UK), 5% FBS (HyClone), 2 mM L-glutamine, 72 mg·L^{-1} (L)-penicillin, and 100 mg·mL^{-1} (L)-streptomycin (complete medium). Cells at 70–80% confluence were treated for 30 min in a medium, in which FBS was substituted with charcoal stripped FBS (Sigma-Aldrich, Saint Louis, MO, USA), with 17-β-E2 (Sigma-Aldrich) at various concentrations (1, 10 or 100 nM), or in complete medium in the absence (untreated cells) or in the presence of 5 mM *N*-acetylcysteine (NAC, Sigma-Aldrich) for 16 h.

Subsequently, after removal of the media, treated cells were cultured for another 4 or 24 h in serum free medium in the presence of 17-β-E2 or NAC. Untreated cells were cultured for 4 or 24 h in serum free medium (S, starved cells) or in fresh complete medium (C, control). 0.01% ethanol (vehicle for estrogen) was added to all untreated 17-β-E2 cells. For experiments with inhibitors, cells were pretreated for 30 min in complete medium with 1 μM diphenyleneiodonium (DPI) or 100 μM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) or 5 nM 2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl-triphenylphosphonium chloride monohydrate (Mito TEMPO) (Sigma-Aldrich), in the presence or in the absence of estrogen. Subsequently, the complete medium was removed and for another 4 or 24 h, cells were cultured with inhibitors with or without estrogen in serum-free medium. In experiments with DPI, 0.003% DMSO was added to DPI untreated cells. Some treatments were performed in cells transiently transfected with 60 nM mouse GSTP1-1 siRNA corresponding to two DNA target sequences of mouse GSTP1-1 (5′-CCCUCAUCUACA CCAACUA[dT][dT]-3′); 5′-UAGUUGGUGUAGAUGAG GG[dT][dT]-3′) (Sigma) or scrambled siRNA (scr siRNA) (Universal Negative Control #1, Sigma), using lipofectamine RNAiMAX™ (Invitrogen Carlsbad, CA, USA) according to the manufacturer's protocol. The ability of siRNA to silence the expression level of GSTP1-1 mRNA was checked 24 h after transfection.

Measurement of MLO-4Y apoptosis

MLO-4Y seeded in six-well plates and treated with 17-β-E2 as above reported were used to assess apoptosis using the Cell Death Detection ELISA plus Kit (Roche Laboratories, Nutley, NJ, USA), according to the manufacturer's instructions. The apoptosis assay was performed measuring the specific increase in mono- and oligonucleosomes released into the cytoplasmic fractions obtained from 10^4 cells using the following formula: fold-increases = absorbance of the samples/absorbance of the corresponding controls (C).

Measurement of intracellular ROS

The intracellular ROS production was measured in MLO-4Y seeded in six-well plates and treated as above reported. One hour before the end of treatments, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) was added to culture medium. After PBS washing, cells were lysed in RIPA buffer (50 mM Tris/HCL pH 7.5, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 2 mM EGTA; Sigma-Aldrich), centrifuged at 11 600 ***g*** for 10 min, and analyzed immediately by fluorescence spectrophotometric analysis at 510 nm. Data, normalized on total protein content, were expressed as fold-increase over the control values.

Western blot analysis

The phosphorylation of ERK1/2 and JNK, the activation of caspase-3 and the expression of RANKL, OPG, sclerostin, GSTP1-1, and MKP-1 were performed by western blot in MLO-4Y treated as above reported. Cells were lysed in ice cold RIPA buffer containing phosphatase and protease inhibitor cocktails (Sigma) and centrifuged at 11 600 ***g*** for 10 min. Equal amounts of total proteins (40–60 μg) from whole-cell extract were subjected under reducing conditions to SDS/PAGE on 10% gel and electrotransferred to PVDF membrane (GE Healthcare). Proteins were visualized by incubating the membranes with specific primary antibodies: anti-caspase 3 or anti-phospho-ERK 1/2 or anti-phospho-JNK (Cell Signalling Technology, Beverly, MA, USA), or anti-RANKL or anti-OPG or anti-sclerostin or anti-GSTP1-1 or anti-MKP-1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, membranes were stripped and reprobed with anti-ERK1/2 or anti-JNK or anti-β-actin for normalization and densitometric analysis. Secondary antibodies conjugated to horseradish peroxidase were used to detect antigen–antibody complexes with a chemiluminescence reagent kit (Bio-Rad, Hercules, CA, USA). IMAGE J software (National Institutes of Health, Bethesda, MD, USA) was used to perform quantitative analyses, and band values were expressed as percentage relative to values of control.

Immunoprecipitation and immunoblot

Immunoprecipitation was carried out using 2 μg of anti-GSTP1-1 or anti-JNK antibody or negative control mouse IgG (BIOCARE Medical, Pacheco, CA, USA) and 400 μg of total protein lysates, for 16 h at 4 °C. Subsequently, protein A/G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology, Inc.) was added (20 μL) for 1 h at 4–5 °C, after which washes were carried out in PBS supplemented with Tween-100 (0.5%). Precipitate was suspended in sample buffer without β-mercaptoethanol (nonreducing conditions) and was subjected to SDS/PAGE (10%) followed by electrotransfer to PVDF membrane. Reaction with the anti-JNK or anti-GSTP1-1 overnight at 4 °C was carried out. Subsequently to stripping, membranes were reprobed with anti-GSTP1-1 or anti-JNK or anti β–Actin.

Measurement of RANKL and OPG release

Receptor activator kB ligand and OPG release was measured in the culture medium of MLO-Y4 treated as above reported, using quantitative sandwich enzyme immunoassay kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. Data, normalized on total protein content, were expressed as fold-increase over the control values.

Protein assay

Protein concentrations were determined by the bicinchoninic acid solution protein reagent assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard (Sigma-Aldrich).

Statistical analysis

All experiments were performed four times in triplicate. Data were expressed as mean ± SEM and statistical significance of the differences was determined by Student's *t*-test. $P \leq 0.05$ was considered statistically significant.

Results

Effect of 17 β-E2 on apoptosis and ROS production in starved MLO-Y4 cells

Figure 1A shows that apoptosis increased remarkably after 4 and 24 h from the starvation, and the pretreatment of MLO-4Y for 30 min with various concentrations of 17 β-E2 reduced starvation-induced apoptosis at both times, as compared to the respective untreated starved cells**.** Under the experimental conditions in this study, the maximum effect of apoptosis inhibition (about 60%) was measured at 10 nM 17 β-E2, a concentration near physiological value and used in the subsequent treatments. No statistically different change between 10 nM and 100 nM 17 β-E2 was measured. The antiapoptotic effect of 10 nM 17 β-E2 was confirmed by the decrease of caspase-3 active form involved in the apoptosis of osteocytes. In fact, estrogen significantly decreased—by about 50–60%—active 17 kDa caspase-3, that derives from proteolytic cleavage of inactive 32-kDa procaspase-3 [14,21,42] (Fig. 1B). Figure 2 shows that the levels of ROS increased after 4 and 24 h from the starvation, and 17 β-E2 lowered ROS content partially (about 30%) only after 24 h, as compared to untreated starved cells. Subsequently, 17 β-E2 effect on ROS and apoptosis was compared with that obtained in cells treated with: DPI or AEBSF, inhibitors of NADPH oxidase activity; Mito TEMPO, a specific scavenger of mitochondrial ROS; or NAC, a direct antioxidant [14,21,43–45] (Fig. 2A,B). The inhibitors were used at concentrations able to inhibit ROS production. Under our experimental conditions, AEBSF and DPI, similarly to 17 β-E2, significantly reduced ROS levels by about 40% only after 24 h; conversely, Mito TEMPO significantly inhibited ROS levels by about 50–60% at both times, similarly to NAC (Fig. 2A). Figure 2B reports the apoptosis levels measured in cells treated with inhibitors and stimulated or not by 17 β-E2; no change in apoptosis was observed in DPI- and AEBSF-treated cells at either studied time and no synergic effect was detected in cells treated with these inhibitors plus estrogen. Mito TEMPO and NAC decreased apoptosis levels by about 60% as compared with starved cells stimulated or not with estrogen (Fig. 2B).

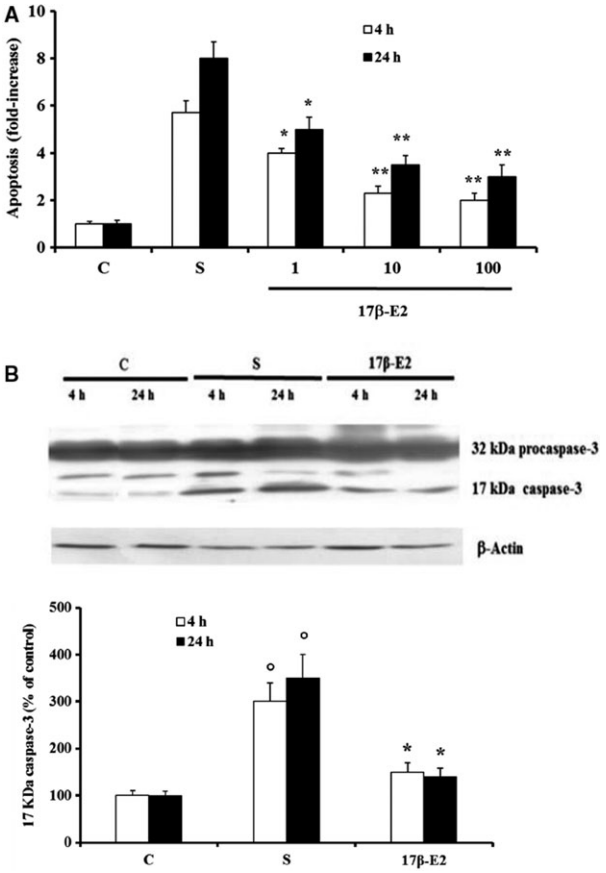

Fig. 1. Effect of various concentrations of 17β-E2 on apoptosis and active 17 KDa caspase-3 in MLO-Y4 cells. Apoptosis (A) and active 17 KDa caspase-3 (B) were measured in MLO-Y4 cells cultured for 4 h and 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 17β-E2 as reported in Materials and methods. Apoptosis data, relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells treated with various nM concentrations of 17β-E2, are expressed as fold-increase over the respective C values and are the mean ± SEM of four experiments. Active 17 KDa caspase-3 was measured in cells treated with 10 nM 17β-E2 by Western blot analysis. Blots are representative of four experiments and the active 17 kDa caspase-3 values are normalized with β-Actin bands obtained by densitometric analysis and reported as the mean percentage ± SEM relative to the respective C values in the bottom. $*P \leq 0.05$ and $**P \leq 0.005$ compared to the respective S values; $^{\circ}P \leq 0.005$ compared to the respective C values.

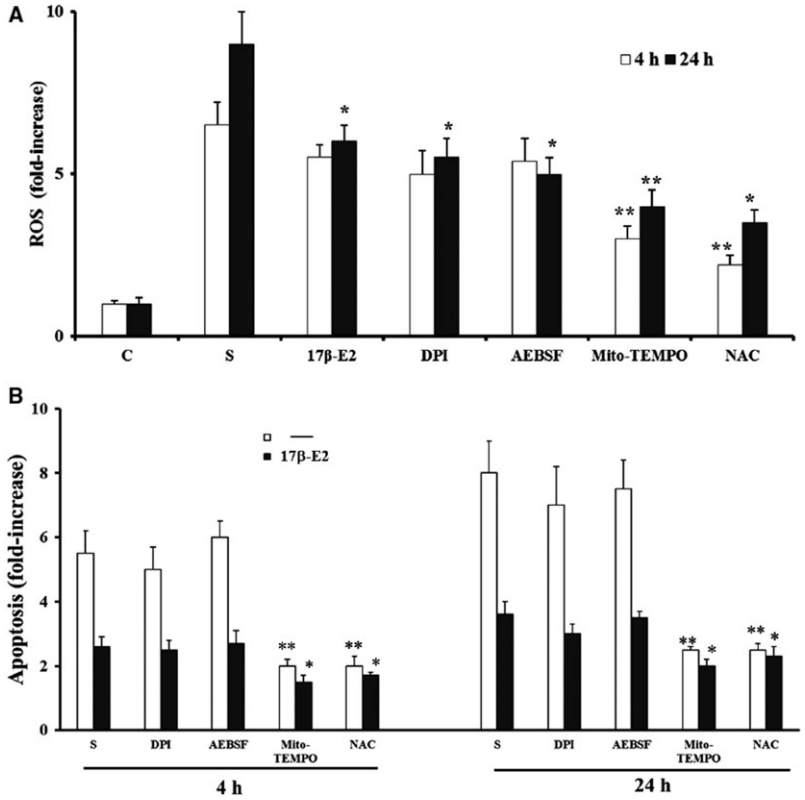

Fig. 2. Effect of 17β-E2, DPI, AEBSF, Mito Tempo and NAC on intracellular ROS and apoptosis in MLO-Y4 cells. Intracellular ROS (A) and apoptosis (B) were measured in MLO-Y4 cells cultured for 4 and 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2, 1 μM DPI, 100 μM AEBSF, 5 nM Mito-TEMPO, or 5 mM NAC as reported in Materials and methods. ROS data, normalized on total protein content, and apoptosis data, relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells, are expressed as fold-increase over the respective C values. The data are the mean ± SEM of four independent experiments. $*P \leq 0.05$ and $**P \leq 0.005$ compared to the respective S values.

Effect of 17 β-E2 on ERK1/2 and JNK activation and MKP-1 expression in starved MLO-Y4 cells

Figure 3 shows that both ERK1/2 and JNK phosphorylation increased after 4 h and 24 h from starvation, and 17 β-E2 inhibited JNK activation at both times by about 60–70%, as compared with unstimulated starved cells (Fig. 3B); no significant effect of 17 β-E2 on ERK1/2 activation was observed (Fig. 3A). Previously, we related starvation-induced apoptosis to JNK activation [21], therefore, in order to clarify the involvement of JNK inhibition on 17 β-E2 antiapoptotic effect, the expression of MKP-1, a specific phosphatase for JNK [32,33], was investigated. Figure 3C shows no change in MKP-1 expression at both times in 17 β-E2 cells, stimulated or not.

Effect of 17 β-E2 and NAC on JNK association with monomeric GSTP1-1 form and GSTP1-1 expression in starved MLO-Y4 cells

Previous data demonstrated that GSTP1-1 monomeric form binds JNK, inhibiting its activation, and the ability of GSTP1-1 to bound JNK is related to oxidative state and/or to GSTP1-1 expression level [34,35,46,47]. In Fig. 4A no band of JNK or GSTP1-1 or β-Actin was detected in Western blot analysis of immunoprecipitates performed with IgG (negative control) in control cells. Differently, JNK and GSTP1-1 bands and no β-Actin were detected after immunoprecipitation with anti-GSTP1-1 or anti-JNK antibody (Fig. 4A). These data demonstrate the absence of non-specific bands under these experimental conditions. Figure 4B shows JNK bands obtained by western blot analysis under nonreducing SDS/PAGE of immunoprecipitates performed with anti-GSTP1-1 antibody; a decrease in JNK associated with monomeric GSTP1-1 form (26 kD) was registered only in starved cells as compared with control. On the contrary, both 17 β-E2 and NAC treatments prevented this dissociation after 4 and 24 h, and the band density of JNK was similar to those of controls (Fig. 4B). The stripping and reprobing of the same blots by anti-GSTP1-1 antibody show that the band density of GSTP1-1 monomeric form changed similarly to that of JNK bands (Fig. 4B). The same results with regard to the association of monomeric form of GSTP1-1 with JNK were obtained by JNK immunoprecipitation experiments performed under nonreducing SDS/PAGE (Fig. 4C).

To evaluate the expression of total GSTP1-1 in 17 β-E2- and NAC-treated cells, western blot analysis of cellular lysates in reducing conditions was performed (Fig. 4D). A significant increase in total GSTP1-1 expression in estrogen-treated cells, as compared to control, was measured but no variation was noted in starved and NAC-treated cells.

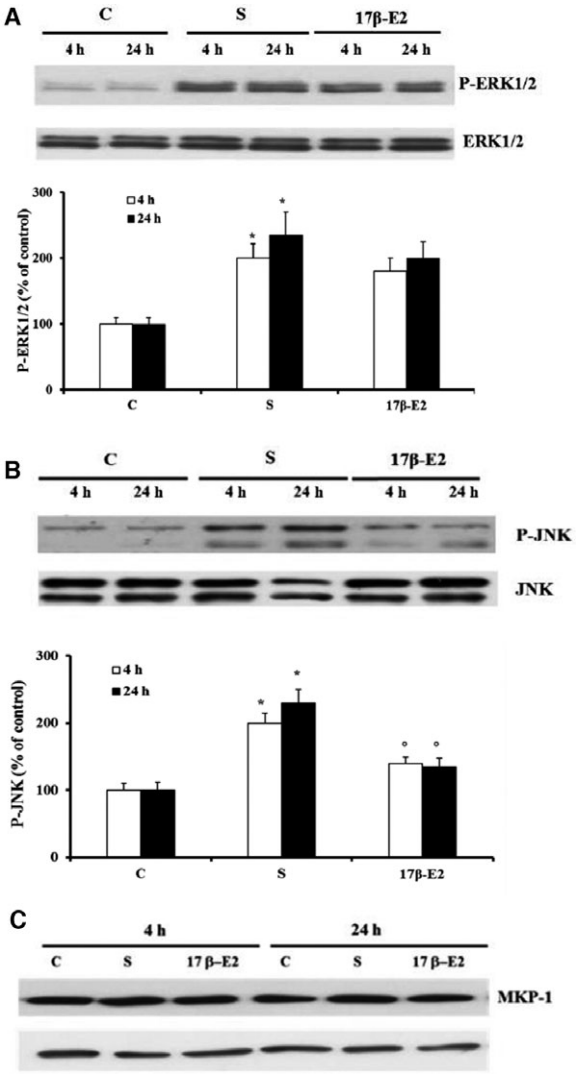

Fig. 3. Effect of 17β-E2 on MAPKs phosphorylation and MKP-1 expression in MLO-4Y cells. Phosphorylation of ERK1/2 (A) and JNK (B) and MKP-1 expression (C) were detected by western blot analysis in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2 as reported in Materials and methods. Blots are representative of four experiments and the values are normalized with ERK1/2 or JNK bands obtained by densitometric analysis and reported as mean percentage ± SEM relative to the respective C values in the bottom. $^{*}P \leq 0.05$ compared to the respective C values; $^{\circ}P \leq 0.05$ as compared to the respective S values.

Role of GSTP1 in 17 β-E2 and NAC effect on apoptosis and JNK activation in starved MLO-Y4 cells

Figure 5A shows that GSTP1-1 expression decreased in control cells transfected with specific siRNA after 24 h of transfection. Figure 5B shows that the down-regulation of GSTP1-1 induced a significant increase in apoptosis in starved cells treated with NAC or 17 β-E2 at both studied times, as compared to the respective scr siRNA-starved cell values (Fig. 5B). In particular, down-regulation of GSTP1-1 in 17 β-E2-treated cells reverted the apoptosis and JNK activation levels to the values measured in untreated scr siRNA starved cells (Fig. 5B–D). In NAC-treated cells, down-regulation of GSTP1-1 increased also apoptosis and JNK activation levels, but these did not reach the levels of untreated scr siRNA starved cell, even if the changes in JNK activation were not significant as compared with untreated scr siRNA-starved cells (Fig. 5B–D).

Figure 6 summarizes the possible effects of 17 β-E2 and NAC on the association of JNK to GSTP1-1 in relation to JNK and apoptosis activation in starved MLO-4Y cells.

Effect of 17 β-E2 on expression and release of RANKL and OPG, and on Sclerostin expression in starved MLO-Y4 cells

The expression of RANKL, OPG, and sclerostin was determined in starved MLO-4Y cells treated with 17 β-E2, considering that in these cells a relationship among apoptosis, increased oxidative state, and expression of these factors has been previously found [21]. In particular, in the cited study, in starved MLO-Y4 cells oxidative stress-induced activation of JNK and ERK1/2 and this was related to RANKL and sclerostin up-regulation, whereas only JNK activation was involved in decreased OPG levels [21]. Figure 7A,B shows, after 4 or 24 h of starvation, a significant increase in RANKL and sclerostin levels, as compared with control, and 17 β-E2 treatment decreased them by about 50%-60%, but levels did not return to those of controls. On the contrary, OPG remarkably decreased at both starvation times, as compared with control cells, and 17 β-E2 inhibited by about 50% the reduction of OPG expression only after 24 h of starvation (Fig. 7C). Also, in this case estrogen was not able to revert OPG levels to the control value (Fig. 7C). Figure 7D shows that 17 β-E2 treatment significantly lowered RANKL release after 24 h of starvation as compared with untreated and starved cells. However, 17 β-E2 did not restore RANKL release to control values in accordance to that observed on RANKL expression. Similar results were obtained after 4 h of starvation (data not shown). Regarding OPG protein release, we were not able to measure the levels of this protein by ELISA kit in MLO-Y4 in agreement with results previously reported by us and others [21,31]. For this reason, the RANKL/OPG ratio was measured by

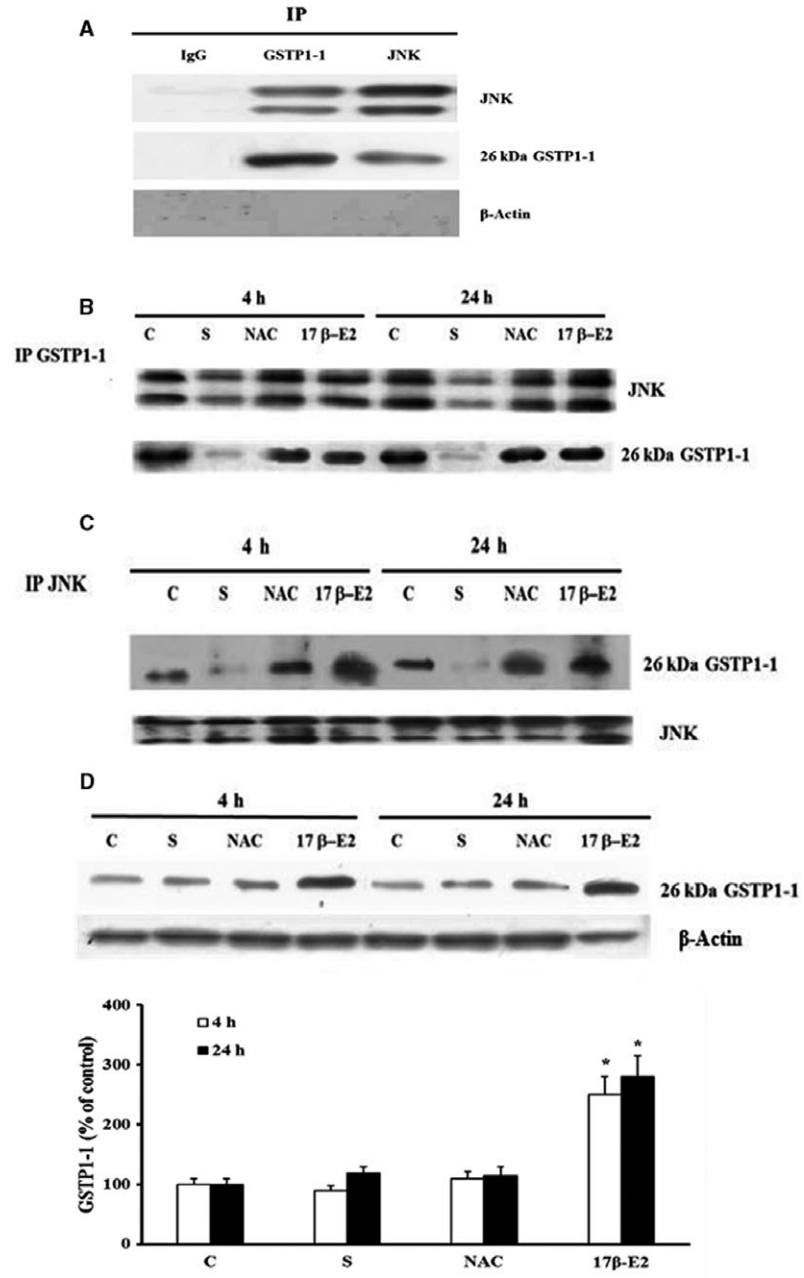

Fig. 4. Effect of 17β-E2 and NAC on JNK association with monomeric GSTP1-1 form and GSTP1-1 expression in MLO-4Y cells. JNK association with GSTP1-1 and GSTP1-1 expression were detected in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2 or 5 mM NAC as reported in Materials and methods. The negative control was performed in MLO-Y4 cells cultured for 24 h in complete medium by immunoprecipitation with negative control mouse IgG (A). For detection of JNK bound to-GSTP1-1 or IgG (A and B), or GSTP1-1 bound to JNK or IgG (A and C), or βActin bound to IgG (A) all immunoprecipitates of equal proteins (400 μg) were performed using anti-GSTP1-1 antibody or anti-JNK antibody or IgG respectively, as reported in Materials and Methods. Western blot analyses were performed under non-reducing conditions (without β-mercaptoethanol) and membranes were probed with anti-JNK (A and B) or anti GSTP1-1 (C) antibodies and subsequently, after stripping, with anti-GSTP1-1 (A and B) or anti-JNK (C) or anti-βActin (A) antibodies, respectively. GSTP1-1 expression (D) was detected by Western blot analysis performed under reducing condition. The normalized values with β-Actin bands obtained by densitometric analysis are reported as mean percentage ± SEM relative to the respective C values in the bottom. The blots are representative of four experiments. *$P \leq 0.05$ compared to the respective C values.

the expression values obtained in western blot analysis (Fig. 7A,C). The ratio increased in a similar manner after 4 h and 24 h of starvation as compared with control cells (Fig. 7E), and 17 β-E2 significantly lowered the ratio values at both times by about 40–70%, respectively (Fig. 7E). The decrease in ratio value after 24 h of starvation was significantly higher than that obtained after 4 h.

Discussion

This study provides new evidence that 17β-E2 in starved MLO-Y4 quickly reduces apoptosis and the related caspase-3 activation due to increased oxidative state, without significant inhibition of ROS production. Under the same experimental conditions, 17β-E2 prevents the increase in RANKL and sclerostin expression, RANKL release, and RANKL/OPG ratio. For the first time, these effects of 17β-E2 are shown to occur by a redox-independent process of JNK activation involving GSTP1-1 overexpression. The ability of 17β-E2 to prevent starvation-induced apoptosis is similar to that obtained in MLO-Y4 after treatment with the antioxidants [21], indeed, we previously demonstrated a redox-regulated osteocyte apoptosis due to

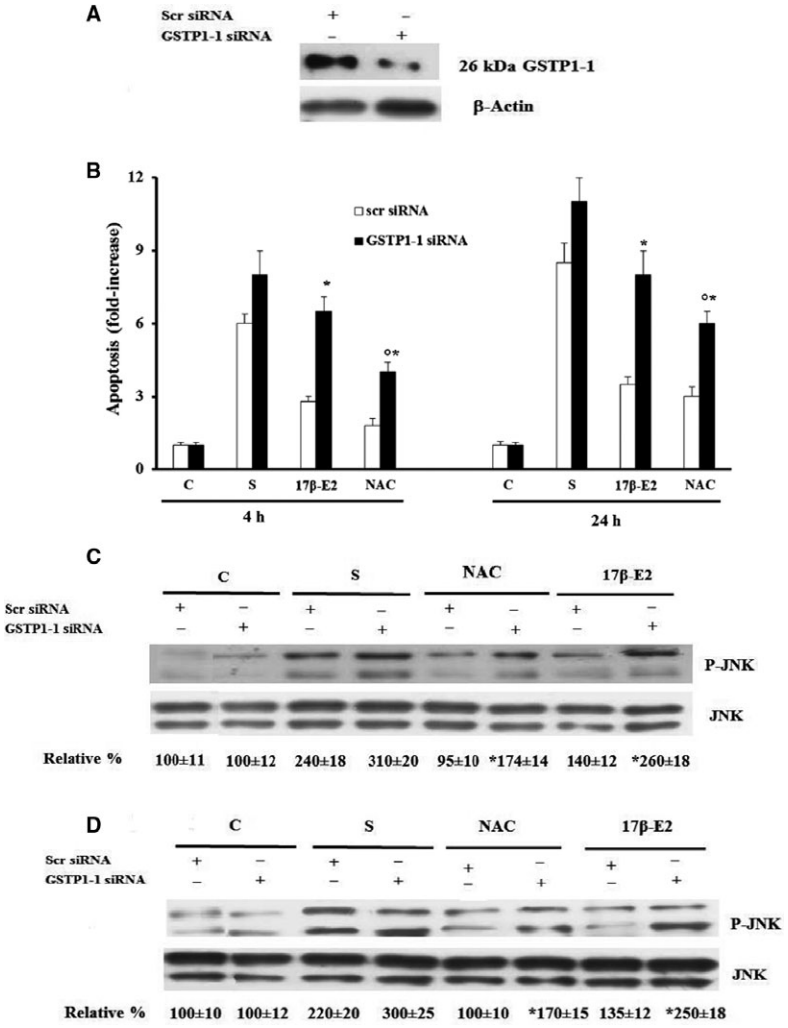

Fig. 5. Effect of GSTP1-1 siRNA on apoptosis and JNK phosphorylation in 17β-E2 or NAC treated MLO-4Y cells. MLO-Y4 cells, transfected with GSTP1-1 siRNA or scr siRNA (negative control) for 24 h, were cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). Transfected S were treated or not with 10 nM 17 β -E2 or 5 mM NAC as reported in Materials and methods. GSTP1-1 expression (A) and JNK phosphorylation after 4 h (C) or 24 h (D) were detected by western blot analysis. The blots are representative of three experiments and the normalized values with JNK bands obtained by densitometric analysis are reported as mean percentage ± SEM relative to the respective C values. Apoptosis data (B), relative to mono- and oligonucleosomes released into the cytoplasmic fraction from 10^4 cells, are expressed as fold-increase over C values, and are the mean ± SEM of three independent experiments. *$P \leq 0.05$ compared to the respective scr siRNA-treated cell values. $^{\circ}P \leq 0.05$ compared to scr siRNA S values.

starvation. However, our data in MLO-Y4 demonstrate that 17β-E2 significantly decreases starvation-induced apoptosis and caspase-3 activation at both the studied times, but only after 24 h from the starvation does 17β-E2 show a weak antioxidant action. On the contrary, NAC, a direct ROS scavenger, remarkably reduces ROS levels at both times, and this has previously been related to its ability to prevent starvation-induced osteocyte apoptosis [21]. Some authors demonstrate that the antiapoptotic effect of estrogen is due to its action as direct ROS scavenger [14] and others indicate 17β-E2 as inhibitor of NADPH oxidase [43]. However, our findings do not show a relationship between the antiapoptotic effect of 17β-E2 and its antioxidant action, which seems to be weak, late, and caused by the inhibition of NADPH oxidase activity, a membrane enzyme which increases cytoplasmic oxidative state [44]. Diversely, in starved osteocytes the increased ROS levels and consequent apoptosis at both times seem to be mainly of mitochondrial origin, and this agrees with previous data which show in MLO-Y4 the involvement of JNK activation [21]. Indeed, JNK activity prevalently mediates stress-induced apoptosis by mitochondrial pathways [48], and this may occur in osteocytes through increased permeability of the outer mitochondrial membrane with the release of cytochrome C-activating caspases (caspase-3,-6 and -7) responsible for cell death [19].

Effectively, it seems difficult for estrogen to have a direct antioxidant effect as shown by others who induced apoptosis by extracellular H_2O_2 treatment in MLO-Y4 cells [14]. In fact, in this study, the estrogen was used at a much lower concentration (10 nM) than that of H_2O_2 (0.3 mM). Also "*in vivo*" it is unlikely that estrogens may have a direct antioxidant effect given that their circulating levels are lower than the concentrations of chemical antioxidants necessary to reduce the oxidative stress [9,49]. Indeed, "*in vitro*"

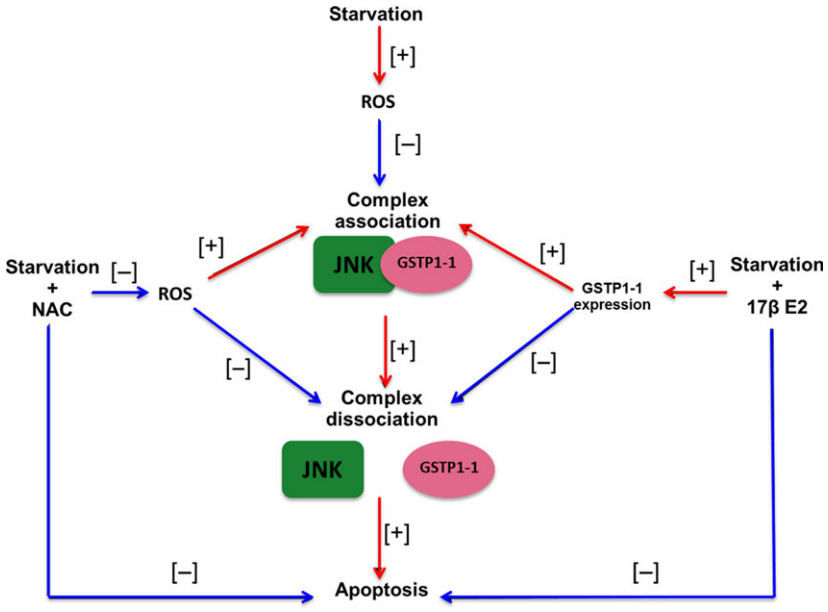

Fig. 6. Summary of 17β-E2 and NAC different mechanisms on JNK-GSTP1-1 complex formation in starved MLO-Y4 cells. NAC and 17β-E2 reduce JNK-GSTP1-1 complex dissociation- and starvation-induced apoptosis favoring JNK/GSTP1-1 complex association by ROS decrease and GSTP1-1 expression increase, respectively. [+] = increase; [–] = decrease

and "*in vivo*" studies demonstrate that 17β-E2 antioxidant action is related to an up-regulation of antioxidant enzymes [9,11–13,49]. However, this mechanism may require longer than a direct antioxidant action, and our data suggest that the early antiapoptotic effect of 17β-E2 occurs in the presence of an oxidative state. As previously reported, JNK and ERK1/2 are activated by an increased oxidative status in starvation-induced osteocyte apoptosis [21]. However, it has been demonstrated that only JNK activity is involved in oxidative stress-induced apoptosis in MLO-Y4, and that the antioxidants including NAC are able to inhibit both JNK and ERK1/2 activity by decreasing ROS levels [21]. Similarly to antioxidants, 17β-E2 inhibits JNK activation in starved MLO-Y4 cells at both studied times, and this can be related to the antiapoptotic effect of 17β-E2, but not to decreased ROS levels. This indicates that 17β-E2 antiapoptotic effect occurs through a process which is unrelated to oxidative status. Moreover, 17β-E2 does not affect ERK1/2 activation due to the presence of the oxidative status, indicating that ERK1/2 activation is not involved in the 17β-E2 protective effect against osteocyte apoptosis, similarly to that observed by others in osteocyte apoptosis induced by H_2O_2 treatment [14].

No data are reported in literature on a protective effect of 17β-E2 on oxidative stress-induced apoptosis in osteocytes by down-regulation of JNK activity. For this purpose, we studied the role of MKP-1, a member of the MKP family that predominately dephosphorylates JNK [32,33], in estrogen-induced JNK inactivation. Indeed, through its receptors 17β-E2 can induce genomic expression of MKP-1 in various cells including bone cells [32], and it has been shown that MKP-1-modulated JNK activity is critical for apoptosis induced by the inhibition of epidermal growth factor receptor tyrosine kinase in lung cancer cells [32]. We demonstrated that MKP-1 is expressed in osteocytes but, under the studied experimental conditions, 17β-E2 and the increased oxidative state do not induce an overexpression of this phosphatase, excluding its possible role in JNK inactivation by estrogen.

Our findings demonstrate, for the first time, that GSTP1-1, a detoxifying enzyme also involved in cell death and/or survival mechanisms, is expressed in osteocytes and is related to the antiapoptotic effect of 17β-E2 mediated by JNK inactivation in the presence of oxidative state. In fact, it is well demonstrated that GSTP1-1 is a regulator of JNK signaling pathways through the formation of a complex with c-Jun-JNK. This association occurs with GSTP1-1 monomeric form and selectively inhibits the phosphorylation and activation of JNK [34,35,46,47,50]. The present work demonstrates that, under normal physiological conditions, GSTP1-1 monomeric form is in reversible equilibrium with dimeric and polymeric forms and that this balance shifts, in the presence of oxidative state, versus polymeric forms due to the formation of disulfide bonds [47,48,50], inducing GSTP1-1/JNK dissociation and apoptosis activation [34,35,46,47]. In agreement with literature data, immunoprecipitation and immunoblot experiments demonstrate that in MLO-4Y cells JNK is bound to GSTP1-1 and under conditions of oxidative stress both the levels of bound

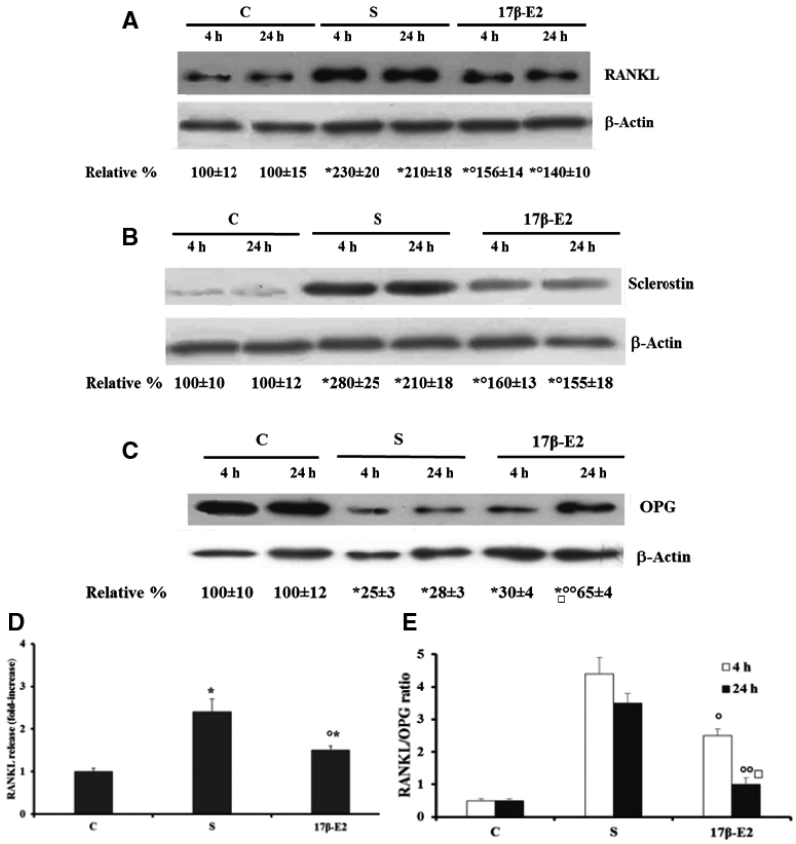

Fig. 7. Effect of 17β-E2 on RANKL, sclerostin, and OPG expression and RANKL release in MLO-Y4 cells. RANKL, sclerostin, and OPG expression and RANKL release were measured in MLO-Y4 cells cultured for 4 or 24 h in complete medium (C, control) or in serum-free medium (S, starved cells). S were treated or not with 10 nM 17β-E2 as reported in Materials and methods. RANKL (A), sclerostin (B), and OPG (C) were measured by western blot analyses of cell lysates. Blots are representative of four experiments and the normalized values with β-Actin bands obtained by densitometric analysis are reported as mean percentage ± SEM relative to the respective C values. RANKL release (D) was detected by immunoenzymatic method in 24 h culture medium, and the values are expressed as fold-increase over the control values. (E) RANKL/OPG ratio was obtained by the expression values measured with western blots. The data are the mean ± SEM of four independent experiments. $^{*}P \leq 0.05$ compared to the respective C values; $^{\circ}P \leq 0.05$ and $^{\circ\circ}P \leq 0.005$ compared to the respective S values; $^{\square}P \leq 0.005$ compared to S values for 4 h and stimulated with 17β-E2.

JNK and GSTP1-1 monomeric form decrease in starved cells. This is inversely related to JNK activation which increases only when there are high levels of ROS, suggesting GSTP1-1/JNK dissociation. This redox regulation of GSTP1-1/JNK complex formation is confirmed by findings obtained with NAC treatment. In fact, NAC preserves JNK bound levels, preventing complex dissociation and JNK activation. This could be due to the ability of NAC to preserve high levels of GSTP1-1 monomeric form through the lowering of ROS levels and the breaking of disulfide bonds present in GSTP1-1 oligomeric forms.

Our data show that 17β-E2 is also able to maintain high levels of both GSTP1-1 monomeric form and JNK linked to GSTP1-1. This indicates that estrogen may preserve GSTP1-1/JNK complex dissociation with the consequent JNK inactivation even if an oxidative state is present. The effect of 17β-E2 can be related to the overexpression of GSTP1-1, which is consistent with data showing that the overexpression of GSTP1-1 can affect the balance between the various forms of GSTP1-1 and the possible bond with JNK [34,35,46,47,51]. Indeed, the increased GSTP1-1 expression can promote the formation of the GSTP1-1/JNK complex by counteracting the action of ROS which, differently, induce complex dissociation and JNK activation. This has been demonstrated by other researchers who have correlated it to apoptosis resistance of tumor cells [34,46,47]. In agreement with our data, Bartolini *et al.* [51] report that GSTP1-1 overexpression inhibits stress-induced JNK activation, whereas it does not affect ERK1/2 activation.

Direct involvement of GSTP1-1 in the protective effect of 17β-E2 and NAC on oxidative stress-induced apoptosis and on their ability to modulate JNK activation has been shown by GSTP1-1 down-regulation. In particular, GSTP1-1 seems important in mediating the early antiapoptotic effect of estrogen through inhibition of JNK in the presence of an oxidative state. In fact, in 17β-E2-treated cells, down-regulation of GSTP1-1 similarly reverts both apoptosis and JNK activation to starved cell values. Also in NAC-treated cells, down-regulation of GSTP1-1 increased both apoptosis and JNK activation levels, but these do not return to starved cell values, indicating that stress-induced JNK activation, followed by apoptosis, may also in part depend on other GSTP1-1-independent redox-regulated factors.

Previously, we demonstrated that when there is an oxidative state in MLO-Y4 the expression of factors, such as RANKL, OPG, and sclerostin involved in bone remodeling, are related to apoptosis as well as to JNK and ERK1/2 activity [21]. Indeed, these factors are expressed by osteocytes under various conditions, including bone pathological alterations [1–4,7,11,52,53]. RANKL increases osteoclast differentiation and bone resorption, whereas OPG competes with RANKL for its receptor inhibiting osteoclastogenesis [2–4,52,53]. Moreover, it has been shown that regulation of the RANKL/OPG ratio is one of the means by which bone resorption and formation can be maintained in equilibrium, and the RANKL/OPG ratio is indicative of osteoclastogenic activity in various bone remodeling diseases [1,3,52,53]. Sclerostin (mainly produced by mature osteocytes) is a negative regulator of osteoblast activity and OPG production [1–5,53] and it inhibits the Wnt/beta-catenin signaling pathway preventing OPG production [1–3].

17β-E2 is able to prevent the increased expression and release of RANKL and the increased expression of sclerostin. However, 17β-E2 is not able to completely revert RANKL and sclerostin levels to those of control, unlike antioxidants which in these cells inhibit both JNK and ERK1/2 activity [21]. 17β-E2 is also able to prevent, in part, the remarkable decrease in OPG levels observed in starved osteocytes, similarly to antioxidants, as only JNK activity is involved in the down-regulation of OPG expression [21]. Given that both estrogen and antioxidants almost totally inhibit JNK activity, their inability to restore normal levels of OPG expression can be correlated with the involvement of other JNK- and/or oxidative stress-independent factors and likely related to starvation. All these data show that the 17β-E2 effect on expression of the studied cytokines is mainly mediated by JNK inhibition. 17β-E2 remarkably decreases RANKL/OPG ratio after 24 h from starvation because only at this time 17β-E2 significantly increases OPG levels. However, 17β-E2, different from that which occurs with antioxidants, does not revert the ratio values to those of the control [21] probably because of the inability of estrogen to quickly eliminate ROS and to inhibit ERK1/2 activation which affects RANKL expression. All these data show that the fast effect of 17β-E2 on the expression of the studied cytokines in the presence of oxidative state depends mainly on GSTP1-1 expression that affects JNK activity. Thereafter, the regulation of GSTP1-1/JNK association may be related to estrogen signaling and RANKL/OPG levels.

These data in osteocytes support the efficacy of estrogen treatment in postmenopausal women by decreasing sclerostin and RANKL levels, which result enhanced in serum during estrogen deficiency [1,2,54]. Indeed, both high levels of RANKL and sclerostin are implicated in the increased bone resorption associated with estrogen deficiency and microdamage [12,19,22,23,52]. The effect of 17β-E2 on the up-regulation of OPG may be related to the down-regulation of sclerostin expression, and this event may be also related to the modulation of JNK activity by up-regulation of GSTP1-1. This agrees with a potential crosstalk between the Wnt/beta-catenin signaling pathway and estrogen signaling [3,4,37].

Conclusions

The present work shows, for the first time in MLO-Y4 osteocyte-like cells, that 17β-E2 prevents early stress-induced osteocyte apoptosis which can occur "*in vivo*" in estrogen deficiency by inhibition of JNK activation due to an increased oxidative state. A novel and important direct involvement of GSTP1-1 in the 17β-E2 effect on JNK activation and apoptosis in osteocytes has been demonstrated. 17β-E2 up-regulates GSTP1-1 expression, contributing to maintain JNK bound to GSTP1-1 and then to inhibit JNK activity. Our study also shows that estrogen achieves an effect similar to NAC that, through oxidative state elimination, is able to inhibit apoptosis and JNK activity while maintaining JNK bound to GSTP1-1. In fact, others have demonstrated that the formation of GSTP1-1/JNK complex may be regulated by oxidative state changes and/or GSTP1-1 overexpression. It is interesting to note that the antiapoptotic effect of 17β-E2 is not due to a direct and quick antioxidant action. Indeed, antioxidant activity of estrogen can occur later through the increased expression of antioxidant

enzymes as other researchers have demonstrated. Moreover, this study indicates that 17β-E2 through JNK inhibition by GSTP1-1 association can decrease the RANKL/OPG ratio and sclerostin level, factors related to osteogenesis and bone remodeling.

Overall, the data presented here clarify 17β-E2 action at molecular level in osteocyte activity on bone remodeling, and identify a possible role of GSTP1-1 and JNK activity in bone repair mechanisms in pathologies related to oxidative stress. Finally, they confirm the validity of antioxidants as therapeutic support.

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Author contribution

MTV and MLB performed the design of the study, interpreted the results, and participated in the writing and drafting manuscript; FF and VD performed experiments and collected data; GM and TI participated in the design of the study and performed statistical analysis; MTV, MLB, FF, VD, GM, and TI revised manuscript content and provided final approval of the manuscript version to be published.

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Protective role of benzoselenophene derivatives of resveratrol on the induced oxidative stress in intestinal myofibroblasts and osteocytes

Vladana Domazetovic [a], Filippo Fontani [a], Damiano Tanini [b], Veronica D'Esopo [b], Caterina Viglianisi [b], Gemma Marcucci [c], Lucia Panzella [d], Alessandra Napolitano [d], Maria Luisa Brandi [c], Antonella Capperucci [b], Stefano Menichetti [b], Maria Teresa Vincenzini [a], Teresa Iantomasi [a, *]

[a] Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, Viale Morgagni 50, I-50134 Florence, Italy
[b] Department of Chemistry "Ugo Schiff", University of Florence, Via della Lastruccia3-13, I-50019 Sesto Fiorentino, Italy
[c] Department of Surgery and Translational Medicine, Bone Metabolic Diseases Unit, University of Florence, Viale Pieraccini 6, I-50139 Florence, Italy
[d] Department of Chemical Sciences, University of Naples "Federico II", Via Cintia 4, I-80126 Naples, Italy

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ABSTRACT

Resveratrol (RE), a polyphenolic compound present in some food and plants, is characterized by anti-inflammatory and antioxidant properties. However, it is quickly metabolized with consequent loss of its efficacy. In this study, the antioxidant effect of 2-phenyl-benzoselenophene derivatives (VD0, VD1 and VD2) was detected in intestinal myofibroblast and osteocyte cell lines in which the oxidative stress was induced by GSH depletion or starvation, respectively. In fact, the oxidative stress is involved in pathogenesis of inflammatory bowel diseases (IBD) and in increased osteoclastogenesis in osteoporosis. Our results show that these derivatives have major antioxidant power in reducing and/or restoring radical oxygen species to control values than RE itself in both cell types. Moreover, derivatives have different antioxidant capacity in myofibroblasts and in osteocytes and this can be due to different degree of oxidative stress and structural characteristics of these compounds. Some of the synthesized RE analogs have shown anti-bacterial role in IBD and anti-resorptive activity in bone pathologies related to inflammatory and osteoporotic processes. Thus, we suggest benzoselenophene derivatives as good candidates for alternative therapy and/or therapeutic support in these pathologies.

1. Introduction

Resveratrol (RE)[1] (3,5,4′-trihydroxy-*trans*-stilbene) belongs to the family of polyphenolic compounds and is present in the grapes and grape-derived foodstuffs, such as red wine, in some berries and oilseeds (peanut) and in particular plants. In traditional Asian medicine the plant Polygonum cuspidatum, particularly rich in RE, is used to treat disorders of the heart and liver [1–3]. RE has attracted much interest considering its protective effects against metabolic and immune-mediated pathologies, as well as its many beneficial properties in treating cancer, cardiovascular diseases, bacterial infections, inflammation and aging [4,5]. RE exerts its anti-inflammatory and anti-autoimmune properties by interacting with signaling molecules, transcriptional factors and various immune cell types [6–11]. In fact, RE ameliorates experimentally induced inflammatory arthritis, ulcerative colitis, autoimmune myocarditis and encephalomyelitis in animal models [12–15]. The beneficial effects of RE are also ascribed to its antioxidant properties. RE induces various intracellular antioxidant enzymes [16], prevents the LDL oxidation [17] and scavenges reactive oxygen species (ROS) protecting biological macromolecules from oxidative damage [18]. ROS are involved in inflammatory processes and in various diseases such as inflammatory bowel disease (IBD), cardiovascular disease and cancer [18,19]. In particular, in IBD a crucial role is attributed to antioxidants and it has been demonstrated that

* Corresponding author. University of Florence, Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", Viale Morgagni, 5, 50134 Firenze, Italy.
E-mail address: tiantomasi@unifi.it (T. Iantomasi).

[1] *Abbreviations:* RE, Resveratrol; ROS, reactive oxygen species; IBD, inflammatory bowel disease; GPx, glutathione peroxidase; GSH, glutathione; BSO, buthionine sulfoximine; H_2DCF-DA, 2′-7′-dichlorodihydrofluorescein diacetate; CD, Crohn's disease; Ph_2Se_2, diphenyl diselenide.

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RE, by reducing the oxidative stress, can be considered an important compound to use in the IBD therapies [20,21]. Oxidative stress, due to estrogen deficiency condition or inflammatory bone disorders [22], is involved in bone loss by contributing to osteoporosis and bone resorption [23]. Antioxidants protect against these events and RE affects bone cell activity by inducing osteoblastogenesis and by inhibiting osteoclast activation [23,24]. The antioxidant activity of RE is due to the presence of phenolic hydroxyl groups and their H^*-transfer ability [25]; however, this effect is not comparable to that of other natural phenols. Moreover, considering that RE is rapidly metabolized with consequent loss of its efficacy, RE derivatives with more powerful antioxidant and anti-inflammatory properties have been synthesized [18,26,27].

It is well known that selenium plays a crucial role in antioxidant system and selenium-containing compounds are essential for biological activities. In this context, organoselenium compounds have continued to attract considerable attention for their essential role in many biological processes, showing for instance antioxidant, anti-inflammatory, and neuroprotective properties [28–30]. Moreover, for their redox chemistry, selenium-containing organic molecules have been widely applied as synthetic mimics of glutathione peroxidase (GPx) [31,32]. GPx is, together with iodothyronine deiodinase and thioredoxin reductase, one of the most important and most studied mammalian selenoenzymes. GPx plays a key role in the cellular defence against oxidative stress, catalyzing the reduction of hydroperoxides at the expense of two molecules of glutathione (GSH). In this regard, selenium-based functionalization of phenolic compounds has been applied to improve the antioxidant potency of such molecules by decreasing the bond dissociation enthalpy of phenolic groups [33], with the aim to conjugate the GPx-like activity with the chain-breaking properties. In particular, Tanini et al. [34] have synthesized three selenated RE derivatives with an increased rigidity, obtained by the construction of a benzoselenophene ring, see Fig. 1 compounds VD0, VD1 and VD2. These RE derivatives were more efficient than RE in ferric reducing/antioxidant power assay and in chain breaking ability evaluation using 'in vitro' styrene autoxidation experiments. Preliminary results showed that these novel selenium-containing RE derivatives proved to behave as GPx-mimics, being able to efficiently oxidize GSH in the presence of H_2O_2. In particular, 150 μM RE benzoselenophene derivatives in the presence of 5 mM GSH reduced 2 mM H_2O_2 in phosphate buffer (pH 7.4) and the highest catalytic activity was found for VD0. This compound indeed showed a T_{50} value (8 min) shorter than diphenyl diselenide (Ph_2Se_2) (16 min) used as reference GPx-mimic, and an activity value of 3.3 as compared to the activity of control with no additive (value = 1) and to that of Ph_2Se_2-treatment (value = 1.6) [unpublished observations].

In this study, we compared RE effects to that of 2-phenyl-benzoselenophene derivatives, VD0, VD1 and VD2, in countering the oxidative stress, induced in myofibroblast cell line derived from human colonic mucosa (18Co), and in murine osteocyte-like cell line, MLO-Y4. In particular, the antioxidant ability of RE derivatives in restoring the physiological redox state of these cells was studied.

2. Materials and Methods

2.1. Reagents

All common reagents were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA), GE Healthcare (Little Chalfont, Great Britain) and Thermo Scientific (Waltham, Massachusetts, USA), unless specified in the text.

Sigma-Aldrich: Minimum Essential Medium Eagle, L-glutamine, sodium bicarbonate, non-essential amino acids, sodium pyruvate, dimethyl sulfoxide (DMSO), buthionine sulfoximine (BSO), trypsin, bovine serum albumin, Tris/HCl, Triton X100, NaCl, NaF, Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA).

GE Healthcare: fetal bovine serum, calf serum, penicillin/streptomycin 100X solution, phosphate buffered saline (PBS), alpha-MEM medium.

Thermo Scientific: Pierce BCA protein assay kit, trypan blue.

2.2. Cell culture and treatment

CCD-18Co (18Co) cells, obtained from American Type Culture

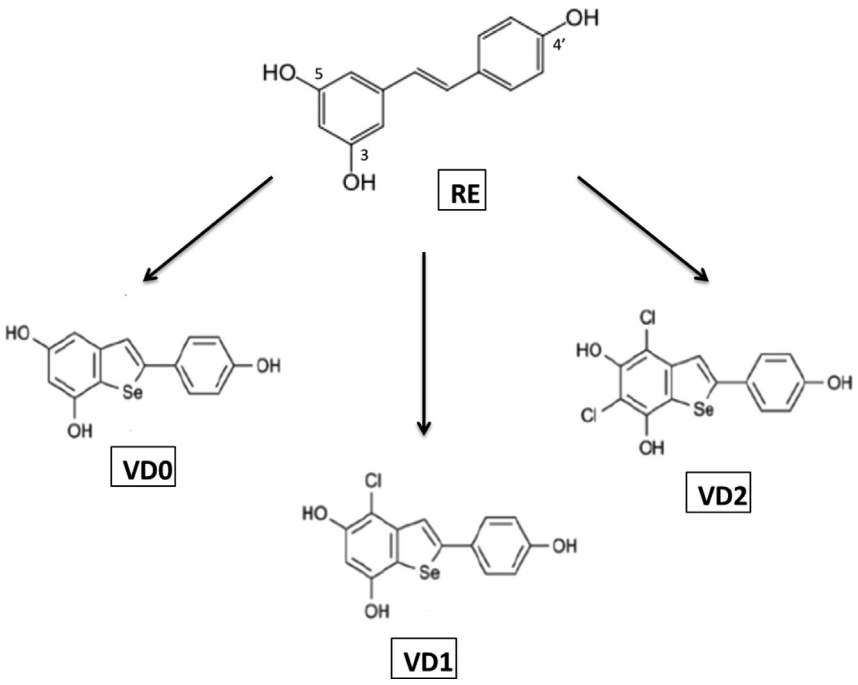

Fig. 1. Chemical structure of RE and benzoselenophene derivatives (VD0, VD1, VD2).

Collection (Manassas, VA), were used in our experiments with PDL 27–36, given that the line begins to senescence at about PDL = 42. MLO-Y4 osteocyte-like cells were a gift from Dr. Lynda Bonewald, University of Missouri-Kansas City. 18Co cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in Minimum Essential Medium Eagle with 2 mM L-glutamine and 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. MLO-Y4 were cultured at 37 °C in a 5% CO_2 humidified atmosphere in alpha-MEM medium supplemented with 5% Calf Serum, 5% Fetal Bovine Serum, 2 mM L-glutamine. Both culture media were supplemented with 72 mg/l penicillin and 100 mg/ml streptomycin. The oxidative stress was induced by treating serum-starved 18Co cells with BSO, an inhibitor of GSH synthesis [19,35]. 18Co cells were starved for 24 h and treated or not during the last 16 h with 25 μM BSO. Differently, considering that starvation is able to induce ROS production in MLO-Y4 [36], the oxidative stress was induced by serum starvation for 24 h in these cells. Subsequently, BSO-treated 18Co and starved MLO-Y4 were treated or not for other 3 h (short-term study) or 24 h (long-term study) with various concentrations (2.5–200 μM) of RE and its derivatives (VD0, VD1 and VD2). Other experiments were performed adding RE and its derivatives during the induction of oxidative stress (prevention study), in particular 1 h prior to 16 h BSO-treated 18Co and 24 h-starved MLO-Y4. 24 h-starved 18Co and unstarved MLO-Y4, were considered controls. 0.008% final concentration of DMSO was present in controls and in all treated and untreated cells.

2.3. Cytotoxicity assay

The cytotoxicity of RE and its benzoselenophene derivatives has been detected, by Trypan blue exclusion test, in 24 h-starved 18Co or in unstarved MLO-Y4 treated or not for 24 h with RE or derivatives at the respective highest concentrations used, 50 μM for 18Co cells and 200 μM for MLO-Y4.

2.4. Benzoselenophene derivatives

RE benzoselenophene derivatives VD0, VD1 and VD2 were obtained by direct selenylation of RE with $SeCl_2$ generated in situ by Se and SO_2Cl_2 in dry tetrahydrofuran, as previously described [34]. Modulation of Se/SO_2Cl_2/RE stoichiometry led to the formation of mixtures with different amounts of parent benzoselenophene (VD0), monochloro (VD1) and dichloro (VD2) benzoselenophene derivatives (Fig. 1).

2.5. Intracellular ROS production assay

The intracellular production of ROS was assayed, as previously described [37] in 18Co or MLO-Y4 cells seeded in 12-well plates treated as above described. 30 min before the end of the various treatments, 5 g/l H_2DCF-DA was added in culture medium. After PBS washing cells were lysed in 50 mM Tris/HCl pH 7.5, 1% Triton X100, 150 mM NaCl, 100 mM NaF, 2 mM EGTA and analysed immediately by fluorescence spectrophotometric analysis at 510 nm. Data have been normalized on total protein content and the values were expressed as percent of ROS production measured in controls. Protein concentrations were determined by the bicinchoninic acid (BCA) solution protein reagent assay [38] using bovine serum albumin as standard.

2.6. Statistical analysis

All experiments were carried out six times. Data are expressed as the mean ± SEM and statistical significance of the differences was determined using one-way ANOVA analysis with Bonferroni's multiple comparison test, using the GraphPad Prism Software. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxic effect of RE and its benzoselenophene derivatives in 18Co and MLO-Y4 cells

Fig. 2A and B shows the cell viability detected in 18Co and MLO-Y4 treated whit RE or its benzoselenophene derivatives at the respective highest concentrations used, 50 μM for 18Co and 200 μM for MLO-Y4. Cell viability did not significantly change in the presence of all compounds as compared to that of the respective untreated cells (Fig. 2A and B). Therefore, considering the lack of cytotoxic effects, RE and its derivatives were used at these or at lower concentrations in further experiments.

3.2. Effect of short- and long-term treatment with RE and benzoselenophene derivatives on BSO-induced oxidative stress in 18Co cells

ROS production was measured in the absence or in the presence of various concentrations (2.5–50 μM) of RE or its benzoselenophene derivatives in BSO-treated 18Co cells. In 18Co cells BSO treatment induced a significant increase in ROS production of about

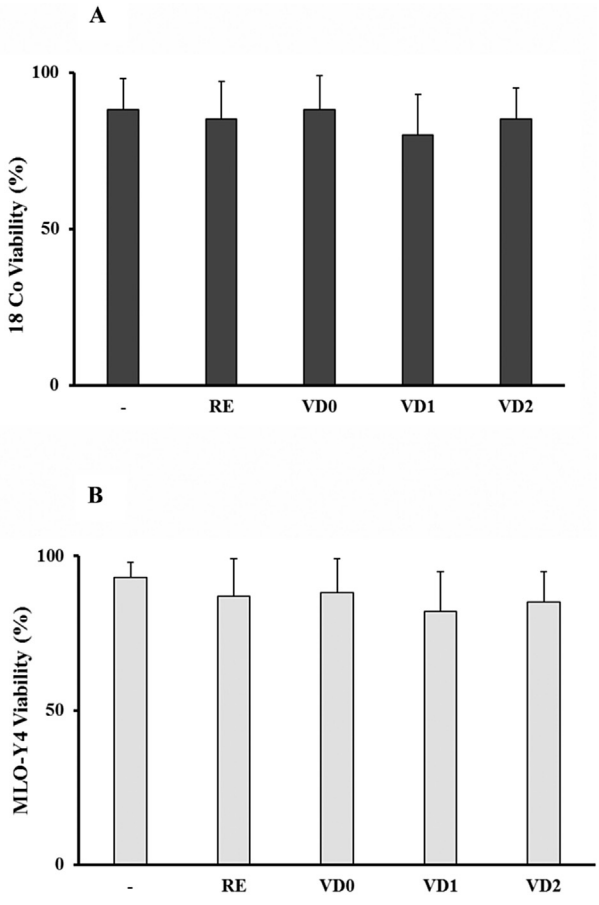

Fig. 2. Cytotoxicity of RE and benzoselenophene derivatives.
Starved 18Co (A) or unstarved MLO-Y4 (B) were treated for 24 h or not with RE or VD0, VD1, VD2 derivatives at 50 μM or 200 μM, respectively. Cell viability was performed by Trypan blue exclusion test and the values, expressed as percent of total cells (living and dead cells), are the mean ± SEM of six experiments in triplicate.

60% in the short-term study (3 h) (Fig. 3A) and of about 100% in long-term study (24 h), as compared to control (Fig. 3B). Fig. 3A shows that the behavior of ROS production following the short-term treatment with RE and its derivatives was mainly concentration-dependent. RE at 10 μM reduced significantly ROS levels restoring them to control values, whereas the same effect was obtained already with 5 μM VD0 and VD1. The highest concentration used of RE and its derivatives (50 μM) decreased significantly ROS levels below those of control (Fig. 3A). This effect was major in derivative-treated cells, even if not significant. An evident concentration-dependence was not obtained in long-term study performed in BSO-treated 18Co cells in the presence of RE and its derivatives (Fig. 3B). In these conditions, among all the studied concentrations of RE, only 50 μM was able to reduce ROS levels restoring them to control values (Fig. 3B). On the contrary, this effect was evident already at the lowest concentration of derivatives (2.5 μM), whereas 50 μM VD0, VD1 and VD2 decreased remarkably ROS levels as compared to control and 50 μM RE. Fig. 3B shows also that ROS production detected with 10 μM VD1 was significantly lower than that of control values, differently to that occurred for VD0 and VD2.

3.3. Effect of short- and long-term treatment with RE and benzoselenophene derivatives on starvation-induced oxidative stress in MLO-4Y cells

Starvation induced a remarkable oxidative stress in MLO-Y4 as indicated by ROS production that increased of about 1300% in the

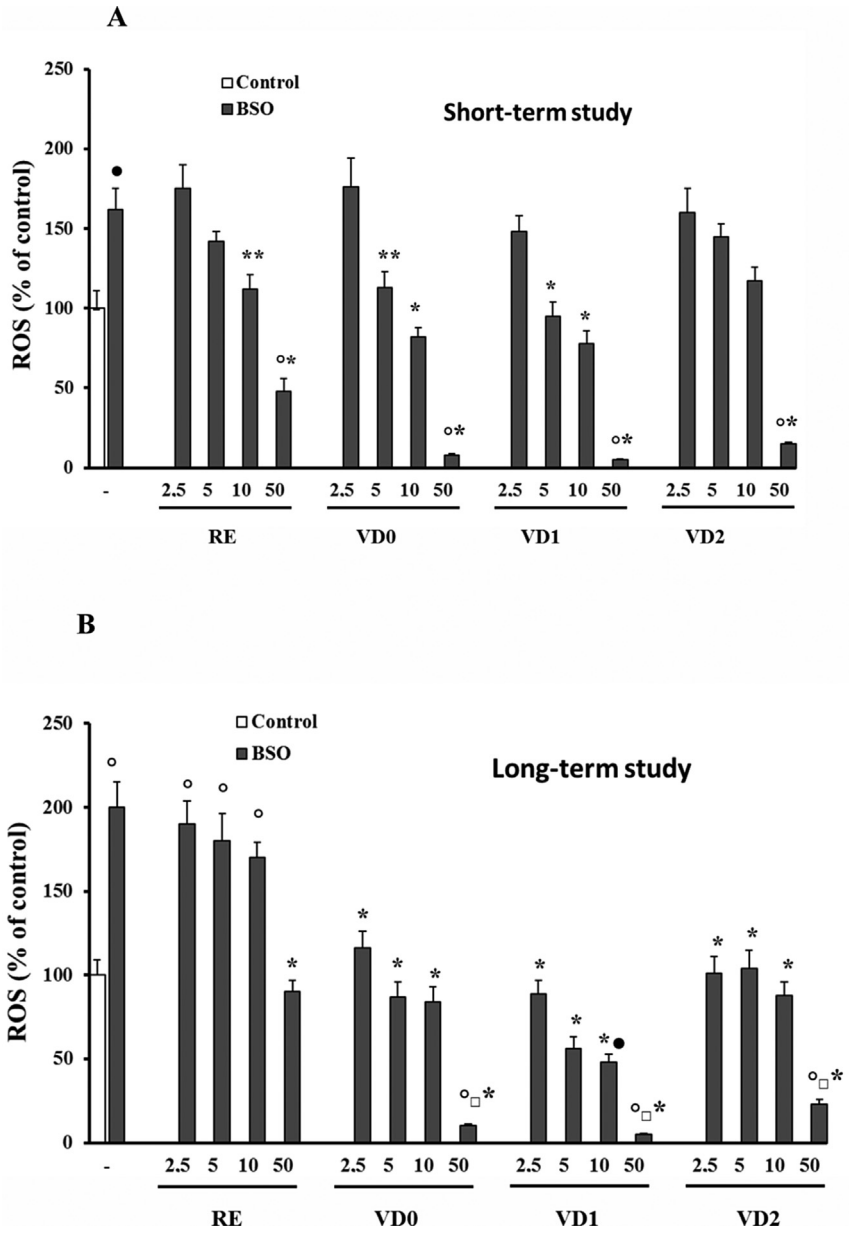

Fig. 3. Intracellular ROS production in BSO-treated 18Co cells after short- and long-term treatment with RE and benzoselenophene derivatives.
BSO-treated 18Co cells were treated or not with various concentrations of RE or VD0, VD1, VD2 derivatives for 3 h (short-term, A) or 24 h (long-term, B) as reported in Materials and Methods. The intracellular ROS production was detected by measuring the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percent of control (BSO-, RE-and derivatives-untreated cells), are the mean ± SEM of six experiments repeated in triplicate.
$^{\circ}p \leq 0.001$ compared to the control 18Co cells; $^{\bullet}p \leq 0.05$ compared to the control 18Co cells; $^{*}p \leq 0.001$ compared to BSO-treated 18Co cells; $^{**}p \leq 0.05$ compared to BSO-treated 18Co cells; $^{\square}p \leq 0.001$ compared to the respective RE-treated 18Co cells.

short-term study (Fig. 4A) and of about 2200% in the long-term study as compared to control (Fig. 4B). Considering this high oxidative stress, we used RE and its benzoselenophene derivatives at higher concentrations ranging from 25 to 200 μM compared to those used in BSO-treated 18Co. Also in starved-MLO-4Y the decrease of ROS level was mainly concentration-dependent after the short-term treatment with RE and its derivatives. All compounds at all concentrations significantly reduced starvation-induced oxidative stress in MLO-Y4 cells (Fig. 4A). In particular, RE and its derivatives VD0 and VD2 in nearly the same way reduced ROS values, as compared to starved and untreated MLO-4Y cells, and no significant difference was detected at concentrations of 100 and 200 μM of these derivatives as compared to control values. On the contrary, VD1 restored ROS to control levels at concentrations ranging from 25 to 200 μM (Fig. 4A). To better compare quantitatively the effect of RE and its derivatives, we performed short-term study treating MLO-Y4 cells with lower concentrations. The insert of Fig. 4A shows the lowest concentrations that reduced significantly ROS levels: 10 μM for RE, VD0 and VD2 and 2.5 μM for VD1. Furthermore, among derivatives only VD1 at all concentrations reduced significantly ROS levels as compared to the respective RE concentrations.

In the long-term study only 100 and 200 μM RE reduced significantly ROS production, as compared to starved-MLO-Y4. It is to note that the value obtained by treating cells with 200 μM RE was approximately similar to values measured with 50 μM benzoselenophene derivatives (Fig. 4B). VD0, VD1 and VD2 at all concentrations used decreased ROS production, as compared to starved MLO-Y4. The trend of this reduction was concentration-dependent, and ROS levels was significantly lower than that measured in cells treated with the respective concentrations of RE. However, VD1 and VD2, at all concentrations used, did not restore ROS levels to control values, differently to that occurred with 200 μM VD0 (Fig. 4B).

3.4. Effect of treatment with RE and benzoselenophene derivatives in the prevention of oxidative stress in 18Co cells and in MLO-Y4

In the prevention study, 18Co cells were treated, 1 h before BSO treatment, with concentrations ranging from 10 to 50 μM for RE and from 2.5 to 10 μM for derivatives (Fig. 5A). RE prevented significantly the oxidative stress only at concentration of 50 μM (Fig. 5A). On the contrary, for derivatives this occurred already at the concentration of 5 μM for VD0 and VD2 and with 2.5 μM for VD1. Prevention study was performed also in MLO-Y4 cells treated with concentrations of RE and derivatives ranging from 25 to 200 μM 1 h prior to starvation (Fig. 5B). RE and derivatives, at all concentrations used, prevented significantly ROS production as compared to starved and untreated cells. Moreover, ROS values, detected in the presence of derivatives, were significantly lower than those measured in RE-treated MLO-Y4 cells. Fig. 5B shows that ROS values were not significantly different as compared to those of control in VD0- (50–200 μM), VD1-and VD2- (100–200 μM) treated cells.

4. Discussion

In this study, the antioxidant effect of RE and its benzoselenophene synthesized derivatives (VD0, VD1 and VD2) was detected in both cell types. The oxidative stress was experimentally induced in 18Co by treatment with BSO, compound normally used to induce GSH deficiency [39], and in MLO-Y4 by starvation. The starvation itself, differently to that occurs in MLO-Y4 cells, does not induce oxidative stress in 18Co cells. These experimental models are useful to create “in vitro” the condition of oxidative stress that characterize pathologies such as IBD or bone inflammatory disorders [19,35,36,40]. In particular, in 18Co cells 25 μM BSO induces an increase of ROS levels similar to those present in intestinal subepithelial myofibroblasts isolated from inflamed mucosa of CD patients [19]. In MLO-Y4 cells starvation mimics “in vitro” a condition similar to that occurs “in vivo” after microdamage or estrogen loss [36,40,41].

18Co cells have many properties similar to those of intestinal subepithelial myofibroblasts that have a very important physiological role [42] and in the presence of oxidative stress can be responsible of an excessive and uncontrolled inflammation as occurs in CD, a chronic intestinal pathology [43–45].

MLO-Y4 cells have a similar phenotype and many characteristics of mature osteocytes [46], and represent a good model to study osteocyte apoptosis subsequent to microdamage and bone disease [41]. Osteocytes control bone remodeling, mineralization and are involved in the repair of microdamage and microfractures [47,48]. Excessive oxidative stress, that can occur during various physiopathological conditions has been related to elevated turnover of bone remodeling and bone mass loss in part due to abnormal apoptosis of osteocytes [49–53]. Indeed, oxidative stress affects bone cell differentiation and inhibits mineralization process [36,54–56].

ROS production in starved BSO-treated 18Co is significantly lower than that detected in starved MLO-Y4. The concentrations used of RE and its benzoselenophene derivatives do not have cytotoxic effects in both cell types and their non-toxicity is confirmed by their antioxidant effect.

Literature data show that different resveratrol analogs were synthesized and their effect on oxidative stress is related to their structural characteristics and cell types [18,27,28]. In fact, hydroxylated resveratrol analogs enhance ROS production in cancer cells reducing proliferation and increasing apoptosis [57], while they have remarkably higher antioxidant ability than RE in rat liver microsomes [58]. Tricyclic derivatives of RE containing hydroxy and/or methoxy substituents in determined positions on the phenolic rings show the same or minor antioxidant effect than RE in THP-1, a cell line of human leukemic monocytes [18].

Our results show that RE benzoselenophene derivatives reduce oxidative stress in 18Co and MLO-Y4 cells much more than RE itself, confirming the data previously demonstrated in a non-biological system [34]. In the short-term study, VD0 and VD1, differently to VD2, restore ROS levels to control values at a concentration that is half of that required for RE in 18 Co. On the contrary, in MLO-Y4 cells only VD1 restores ROS levels to control values. Altogether the data obtained in the short-term study indicate that VD1 possesses higher antioxidant power than RE and other derivatives.

In the long-term study all derivatives restore ROS production to control values in 18Co at concentrations 20 times lower than that of RE. In this experimental condition derivatives are more efficient than RE in reducing oxidative stress in ML0-Y4 cells, however restoration of the redox state occurs only with 200 μM VD0. Also in the prevention of the oxidative stress benzoselenophene derivatives are more effective than RE both in 18 Co and MLO-Y4 cells. In fact, in both cells, this preventive effect is complete at concentrations lower than those of RE. Indeed, altogether our data show that derivatives are able to restore the physiological redox state in 18Co and in MLO-Y4 but at different concentrations, and this can be due to degree of oxidative stress, cell type and structural characteristics of compounds.

Considering high antioxidant capacity of RE benzoselenophene derivatives both in the down-regulation and/or prevention of oxidative stress, we suggest the potential use of these compounds to reduce the inflammatory state in IBD or excessive bone resorption in various pathological bone disorders. In fact, given that RE is quickly adsorbed and metabolized [59], and for this can reach

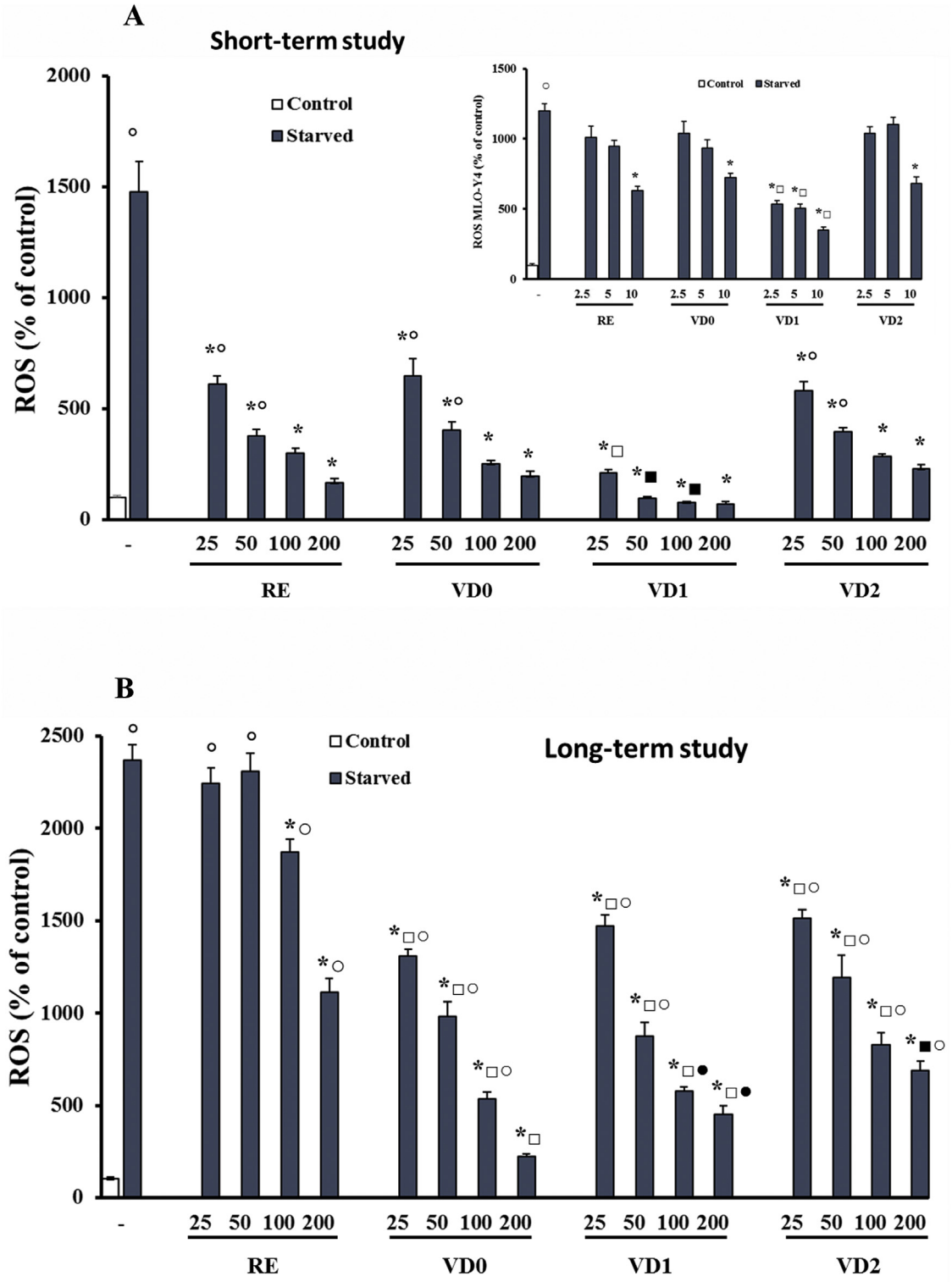

Fig. 4. Intracellular ROS production in starved MLO-Y4 cells after short- and long-term treatment with RE and benzoselenophene derivatives.
Starved MLO-Y4 cells were treated or not with various concentrations of RE or VD0, VD1, VD2 derivatives for 3 h (short-term, A and its insert) or 24 h (long-term, B) as reported in Materials and Methods. The intracellular ROS production was detected by measuring the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percent of control (unstarved and untreated cells), are the mean ± SEM of six experiments repeated in triplicate.
$^{\circ}p \leq 0.001$ compared to the control MLO-Y4 cells; $^{*}p \leq 0.001$ compared to the starved and untreated MLO-Y4 cells; $^{\square}p \leq 0.001$ compared to the respective RE-treated MLO-Y4 cells; $^{\blacksquare}p \leq 0.05$ compared to the respective RE-treated MLO-Y4 cells.

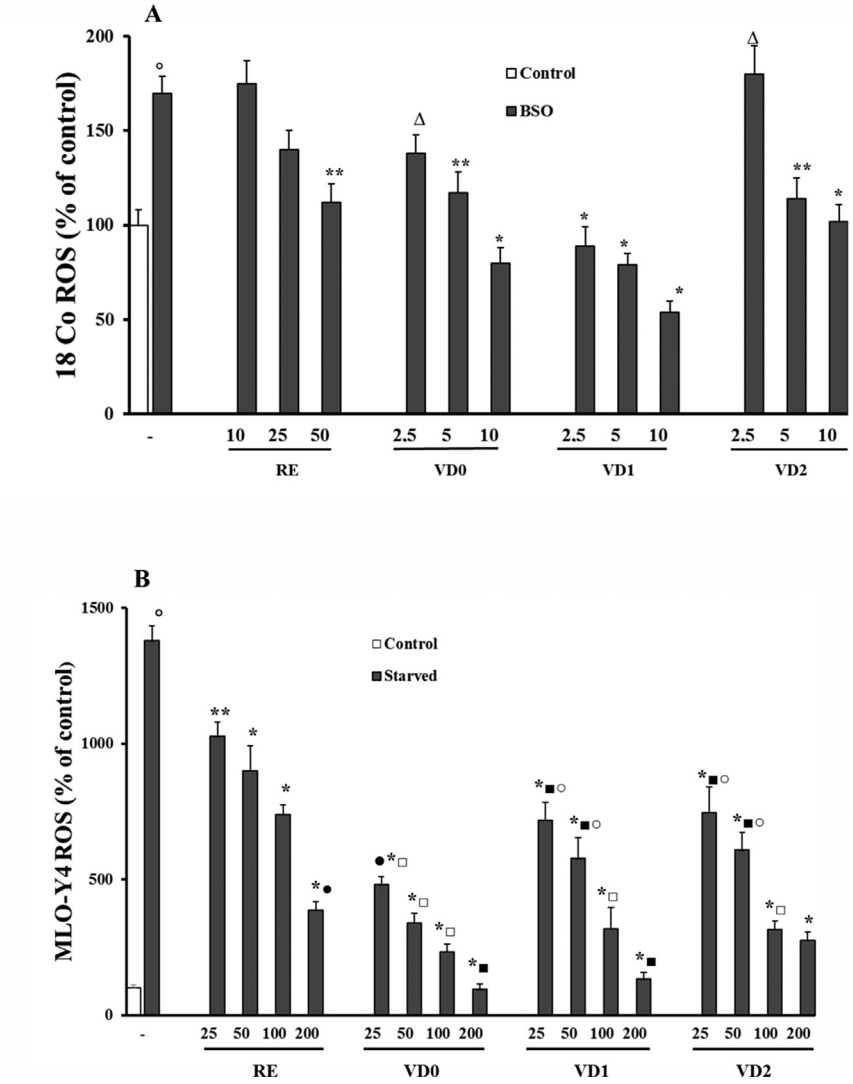

Fig. 5. Intracellular ROS production in BSO-treated 18Co cells and in starved MLO-Y4 cells treated with RE and benzoselenophene derivatives in the prevention of oxidative stress.

Various concentrations of RE or VD0, VD1, VD2 were added or not to 18Co (A) and MLO-Y4 (B) cells 1 h before BSO treatment or starvation, respectively, as reported in Materials and Methods. The intracellular ROS production was detected by measuring the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percent of control (unstarved and BSO-untreated 18Co or unstarved MLO-Y4), are the mean ± SEM of six experiments repeated in triplicate.

$^{\circ}p \leq 0.001$ compared to the control cells; $^{\bullet}p \leq 0.05$ compared to the control cells; $^{*}p \leq 0.001$ compared to BSO-treated 18Co or starved and untreated MLO-Y4 cells; $^{**}p \leq 0.05$ compared to BSO-treated 18Co or starved and untreated MLO-Y4 cells; $^{\square}p \leq 0.001$ compared to the respective RE-treated cells; $^{\blacksquare}p \leq 0.05$ compared to the respective RE-treated cells; $^{\Delta}p \leq 0.05$ compared to the respective VD0-or VD1-treated cells.

hardly the distal part of gut, the possible use of glucosylacyl and glucosyl derivatives of RE has been proposed in IBD. In fact, these compounds block the adherence of pathogens to human colonic cells more efficiently than RE and prevent intestinal inflammation "in vivo" [26,27]. Anti-osteoporotic role of RE has been demonstrated "in vivo" [60] and RE-synthesized oligomers present anti-osteoporotic activity in the ovariectomized female rats [61].

5. Conclusions

This study shows, for the first time, that RE benzoselenophene derivatives have major antioxidant power than RE itself in intestinal myofibroblasts and osteocytes, especially in prevention and in long-term study. Moreover, altogether all derivatives are more effective than RE in restoring physiological redox state. They act at lower concentrations than RE that did not reduce efficiently oxidative stress after the long-term treatment. In particular, VD1 and all derivatives have major antioxidant role in MLO-Y4 after short-term and long-term treatments, respectively. In 18Co cells the highest antioxidant effect has been observed with VD1 during the prevention study. It has been speculated the use of benzoselenophene derivatives as an alternative therapy and/or therapeutic support in intestinal inflammatory disease and osteoporosis. In fact, oxidative stress is responsible of an increased production of inflammatory mediators by intestinal myofibroblasts as well as of enhanced osteoclastogenic factors and bone loss due to osteocytes.

Conflict of interest

The authors have no conflict of interest.

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Transparency document

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ARTICLE
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Tumor Necrosis Factor-Alpha Up-Regulates ICAM-1 Expression and Release in Intestinal Myofibroblasts by Redox-Dependent and -Independent Mechanisms

Filippo Fontani,[1] Vladana Domazetovic,[1] Tommaso Marcucci,[2] Maria Teresa Vincenzini,[1] and Teresa Iantomasi[1]*

[1]*Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, Viale Morgagni 50, 50134 Florence, Italy*

[2]*Santa Maria Annunziata Hospital, Section of General Surgery, 50126 Via dell'Antella 58, Ponte a Niccheri (Florence), Italy*

ABSTRACT

Intercellular adhesion molecule-1 (ICAM-1) is distributed and expressed on cell surface and is present in circulation as soluble form (sICAM-1). Tumor necrosis factor-alpha (TNFα) and radical oxygen species (ROS) up-regulate the expression of ICAM-1. This study demonstrates for the first time in 18 Co cells, a myofibroblast cell line derived from human colonic mucosa, an up-regulation of ICAM-1 expression and sICAM-1 release induced by oxidative stress and TNFα stimulation. The intracellular redox state was modulated by L-buthionine-S,R-sulfoximine (BSO) or N-acetylcysteine (NAC), inhibitor and precursor respectively of GSH synthesis. ROS production increases in cells treated with BSO or TNFα, and this has been related to an up-regulation of ICAM-1 expression and sICAM-1 release. The involvement of metalloproteinases in ICAM-1 release has been demonstrated. Moreover, also expression and activation of A disintegrin and metalloproteinase 17, a membrane-bound enzyme known as TNFα-converting enzyme (TACE), have been related to ROS levels. This suggests the possible involvement of TACE in the cleavage of ICAM-1 on cell surface in condition of oxidative stress. NAC down-regulates the expression and release of ICAM-1 as well as the expression and activation of TACE. However, in TNFα stimulated cells NAC treatment reduces only in part ICAM-1 expression and sICAM-1 release. Given this TNFα may also act on these events by a redox-independent mechanism. J. Cell. Biochem. 117: 370–381, 2016.

KEY WORDS: H_2O_2 PRODUCTION; OXIDATIVE STRESS; ICAM-1 SOLUBLE FORM; REDOX REGULATION; TACE

Intercellular adhesion molecule-1 (ICAM-1) is a glycoprotein extensively distributed and expressed on cell surface of various cell types such as fibroblasts, endothelial and epithelial cells, and leukocytes [Hua, 2013]. The role of ICAM-1 is crucial in immune response inducing the trans-migration of leukocytes to inflammatory sites. ICAM-1 also mediates the intracellular signal transduction pathway, through outside-in signalling event, and the cell-matrix adhesion. Moreover, it promotes the adhesion of cancer cells and is involved in the immune response of tumors [Lawson and Wolf, 2009; Arteta et al., 2010; Ksiazek et al., 2010]. In addition, ICAM-1 is present in circulation as soluble form (sICAM-1), lacking the transmembrane and cytoplasmic domains. sICAM-1 results from proteolytic cleavage of cell surface ICAM-1 through a process that does not depend on the amount of ICAM-1 present on membranes [Lawson and Wolf, 2009; Hua, 2013]. In particular, proteases involved in ICAM-1 cleavage are matrix metalloproteinases (MMPs) [Lawson and Wolf, 2009], A disintegrin and metalloproteinase 17, a membrane-bound enzyme known as tumor necrosis factor-alpha (TNFα) -converting enzyme (TACE), [Tsakadze et al., 2006] and elastase, a serine proteinase secreted by neutrophils and macrophages during inflammation [Champagne et al., 1998]. Although sICAM-1 can have a good therapeutic effect by blocking cell-cell adhesion, it plays a role in chronic inflammatory processes by stimulating the cytokine production [McCabe et al., 1993; Otto et al., 2000], the activation of mitogen activated protein kinases (MAPK) [Otto et al., 2002] and transcriptional factors [Schmal et al., 1998].

An up-regulation and/or over-expression of ICAM-1 and sICAM-1 have been found in tumors, such as melanoma, pancreatic cancer and colorectal cancer (CRC), suggesting a role of ICAM-1 in the malignant progression [Maeda et al., 2002; Roland et al., 2007,2010].

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*Correspondence to: Teresa Iantomasi, Department of Biomedical, Experimental and Clinical Sciences "Mario Serio" Viale Morgagni 50, 50134 Firenze. E-mail: tiantomasi@unifi.it
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In particular, an increase of ICAM-1 on the surface of neoplastic cells mediates the attachment of these cells to the extracellular matrix, with the consequent decrease of metastases and increase of tumor differentiation [Taglia et al., 2007]. On the contrary, sICAM-1 is involved in tumor invasion and in development of metastasis in various cancer types [Nakata et al., 2000; Perabo et al., 2001; Maeda et al., 2002; O'Hanlon et al., 2002].

Pro-inflammatory mediators, cytokines, and radical oxygen species (ROS) up-regulate the expression of ICAM-1 [Roebuck and Finnegan, 1999; Ying et al., 2009; Angel-Morales et al., 2012; Pina-Canseco Mdel et al., 2012]. In fact, an increased expression of ICAM-1 occurs in different pathological inflammatory conditions, such as psoriasis, atherosclerosis, autoimmune diseases and inflammatory bowel diseases (IBD) [Chatterjee, 1998; Metselaar and Storm, 2005; Van Assche and Rutgeerts, 2005]. High levels of ICAM-1 expression have been found in IBD patients and during experimental colitis [Vainer et al., 2000; Chidlow et al., 2006]. In particular, in patients with Crohn's Disease (CD), an enhanced expression of ICAM-1 in the intestinal mucosa has been demonstrated [Ghosh and Panaccione, 2010]. In addition, high serum levels of sICAM-1, which probably derive from the cutting of ICAM-1 from cell surface within gut mucosa, have been measured in CD patients [Nielsen et al., 1994]. High levels of sICAM-1 are also present in serum of patients with CRC [Alexiou et al., 2001] that represents an elevated risk for IBD patients [Feagins et al., 2009; Grivennikov, 2013]. A risk factor for carcinogenesis in IBD patients is the enhanced oxidative state [O'Connor et al., 2010] present in the intestinal mucosa and intestinal subepithelial myofibroblasts (ISEMFs) of these patients [Iantomasi et al., 1994; Buffinton and Doe, 1995; Catarzi et al., 2011a].

ISEMFs, mesenchymal cells localized at interface between enterocytes and lamina propria, regulate various functions of normal and tumoral epithelial cells, stimulate colon tumorigenesis [Pinchuk et al., 2010] and are involved in the colorectal metastases [Yeung et al., 2013]. Moreover, ISEMFs express adhesion molecules, such as ICAM-1 [Pang et al., 1994]. In the literature, to our knowledge, no data are reported on the release of sICAM-1 by these cells and the redox regulation of ICAM-1 and sICAM-1 in ISEMFs.

In this study, we investigated the role of ROS on ICAM-1 expression and sICAM-1 release in myofibroblasts stimulated or not by TNFα. Indeed, TNFα is an important pro-inflammatory mediator related to the regulation of inflammatory signalling pathways in IBD [Biasi et al., 2013]. Moreover, a redox regulated mechanism of TACE expression and activation was also investigated. For this purpose, we performed experiments using a myofibroblast cell line derived from human colonic mucosa, CCD-18Co (18Co) cells which exhibit many properties of ISEMFs [Valentich et al., 1997]. In these cells, the intracellular redox state was modulated by L-buthionine-S,R-sulfoximine (BSO) or N-acetylcysteine (NAC), inhibitor and precursor respectively of GSH synthesis [Catarzi et al., 2011a].

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

CCD-18Co (18Co) cells, obtained from American Type Culture Collection (Manassas, VA), were used in our experiments with PDL 27–36, given that the line begins to senescence at about PDL = 42. The substances used for cell cultures and cell treatments were purchased from Sigma (St. Louis, MO). Cells were cultured at 37°C in a 5% CO_2 atmosphere in minimum essential medium with 2 mM glutamine and 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. Culture media were supplemented with 72 mg/L penicillin and 100 mg/ml streptomycin. Experiments were performed in 18Co cells serum starved at confluence for 24 h and treated or not during the last 16 h with 25 μM BSO or 20 mM NAC or NAC + BSO. Subsequently, the cells were stimulated or not for other 24 h with various concentrations of TNFα (1–30 ng/ml). Additional experiments were performed in 24 h serum starved 18Co cells treated or not, during the last 16 h, with BSO in the presence or absence of 10 μM TNFα-protease inhibitor-1 (TAPI-1) or 10 μM diphenyleneiodonium (DPI). In experiments with DPI, we added 0.003% DMSO (vehicle for DPI) in the respective DPI untreated cells. Subsequently these cells were stimulated for 24 h with TNFα. The concentrations of all compounds used did not reduce the viability according to Trypan blue exclusion test.

INTRACELLULAR ROS PRODUCTION ASSAY

The intracellular production of ROS was assayed, as previously described [Catarzi et al., 2011b], in 18Co cells seeded in 12-well plates, serum starved and treated as above described. Thirty minutes before the end of the various treatments, 5 g/L 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was added in culture medium. After PBS washing, adherent 18Co cells were lysed in 50 mM Tris/HCl pH 7.5, 1% Triton X100, 150 mM NaCl, 100 mM NaF, 2mM EGTA (RIPA buffer) and analysed immediately by fluorescence spectrophotometric analysis at 510 nm. Data have been normalized on total protein content and values were expressed as percent of ROS production measured in untreated and unstimulated cells (control).

ICAM-1 AND sICAM-1 ASSAY

sICAM-1 release and intracellular ICAM-1 levels were measured in cell lysate and in the respective culture medium, using Human ICAM-1 ELISA kit (Uscn Life Sciences Inc., Wuhan, Hubei, PRC) in accordance whit the manufacturer's instructions. 18Co cells were seeded in 12-well plates and treated as above described. For the cell lysates preparation, cells were detached with trypsin and collected by centrifugation at 130*g* for 10 min. Cells, washed three times in cold phosphate buffer saline (PBS), were resuspended in PBS and ultrasonicated for 4 times. Subsequently, the cell lysates were centrifugated at 1,500*g* for 10 min to remove cellular debris and ICAM-1 assay was performed in the supernatants. Data have been normalized on total protein content and sICAM-1 and ICAM-1 levels were expressed as percent of the respective levels measured in control.

WESTERN BLOTTING

ICAM-1 and TACE expression analysis was performed by Western blot in 18Co cells seeded in p-60 plates treated and stimulated or not as above described. Cells were lysed in ice cold RIPA buffer containing phosphatase and protease inhibitor cocktails,

purchased from Sigma and, after 15 min on ice, were centrifuged at 11,600*g* for 10 min. Immunochemical detection of ICAM-1 was performed also in the membrane fraction purified as previously reported [Catarzi et al., 2005]. Protein concentrations were determined by the bicinchoninic acid solution (BCA) protein reagent assay (Pierce) [Smith et al., 1985] using bovine serum albumin as standard (Sigma). Equal amount of total proteins (30–35 μg) were loaded in each line and were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) on 10% (w/v) gel and electrotransferred to PVDF membrane (GE Healtcare) that was probed with specific antibodies anti-ICAM-1 or anti- β-actin (Santa Cruz Biotechnology, INC.). For the detection of TACE and TACE phosphorylated at Thr735, the samples were lyophilized and 100 μg of proteins were subjected to SDS/PAGE on 10% (w/v) gel. PVDF membranes were probed with anti-TACE (Santa Cruz Biotechnology, INC.) or anti- β-actin. Subsequently, the membranes were stripped by incubation for 30 min at 50°C in buffer containing 62.5 mM Tris/HCl, pH 6.7, 100 nM 2-mercaptoethanol, 2% SDS and, after extensive washing, the membranes were reprobed with anti-ADAM17-phospho-Thr735 (MyBioSource). β-actin bands were used to normalize and to perform a densitometric analysis. Secondary antibodies conjugated to horseradish peroxidase were used to detect antigen-antibody complexes with a chemiluminescence reagent kit (GE Healtcare). Chemidoc-Quantity-One software (Biorad Laboratories) was used to perform quantitative analyses and band values were expressed as percentage variations relative to controls.

STATISTICAL ANALYSIS

All experiments were carried out four or more times. Data are expressed as the mean ± SEM and statistical significance of the differences was determined using Student's *t* test. $P \leq 0.05$ was considered statistically significant.

RESULTS

EFFECT OF BSO, NAC, AND TNFα ON INTRACELLULAR ROS LEVELS IN 18Co CELLS

In Figure 1 the intracellular ROS production measured in 18Co cells in different experimental conditions is reported. The cells were treated or not with BSO or NAC or both and, subsequently, stimulated or not with 1 ng/ml TNFα (TNFα 1), physiological concentration [Corredor et al., 2003], and 10 ng/ml TNFα (TNFα 10), concentration at which the maximum effect has been observed. BSO and TNFα increased significantly ROS production in 18Co cells as compared to untreated and unstimulated cells (control) (Fig. 1). Interestingly, TNFα 1 increased ROS levels similarly to that observed in BSO treated cells. Moreover, it enhanced significantly ROS production in BSO treated 18Co cells, as compared to cells stimulated and treated separately with TNFα or BSO, reaching the value obtained with TNFα 10 (Fig. 1). In fact, TNFα 10 enhanced ROS intracellular levels, as compared to TNFα 1 stimulated 18Co cells, and BSO treatment did not make a further increase. Figure 1 shows that NAC treatment reduced significantly ROS levels, as compared to control cells, and this effect was also observed both in BSO treated cells and TNFα 1 stimulated cells. On the contrary, NAC restored ROS levels to control values in all the other conditions (TNFα 10, BSO + TNFα 1, and BSO + TNFα 10).

EFFECT OF BSO, NAC, AND TNFα ON ICAM-1 EXPRESSION AND sICAM-1 RELEASE IN 18Co CELLS

Considering the effects of BSO, NAC, and TNFα on the changes of intracellular oxidative state, under the same experimental conditions, ICAM-1 expression and sICAM-1 release were detected by Western blot analysis of cell lysates and by ELISA kit in culture medium, respectively. Figure 2A shows that ICAM-1 increased both in BSO treated and TNFα stimulated cells, as compared to control. It is noteworthy that the enhancement of ICAM-1 expression was

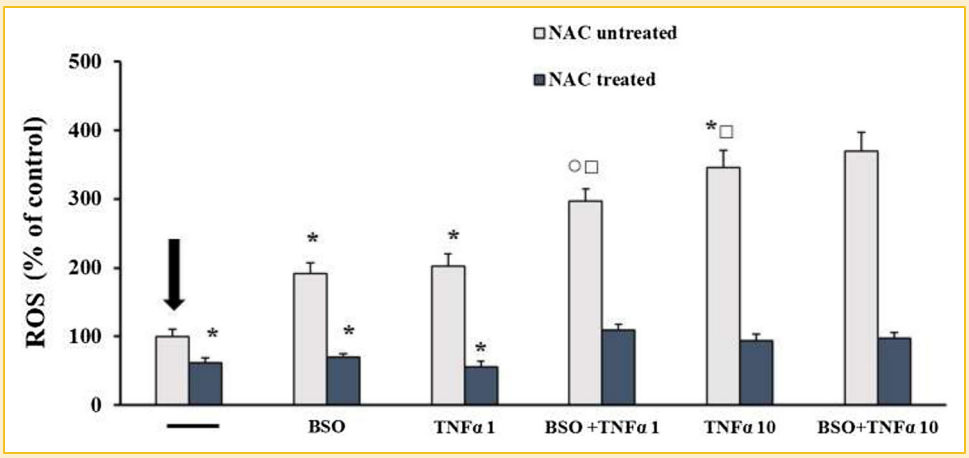

Fig. 1. Intracellular ROS production in 18Co cells treated or not with BSO or NAC and stimulated with TNFα. Starved 18Co cells, treated or not with 25 μM BSO or 20 mM NAC as reported in Materials and Methods, were stimulated or not for 24 h with 1ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). The intracellular ROS production was detected by measuring the fluorescence intensity of the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percent of untreated and unstimulated cells (control, 100%, indicated with the arrow), are the mean ± SEM of six experiments repeated in triplicate. $^{*}P \leq 0.05$ compared to the control cells; $^{\square}P \leq 0.005$ compared to NAC untreated and TNFα1 stimulated cells; $^{\circ}P \leq 0.001$ compared to NAC untreated and BSO treated cells.

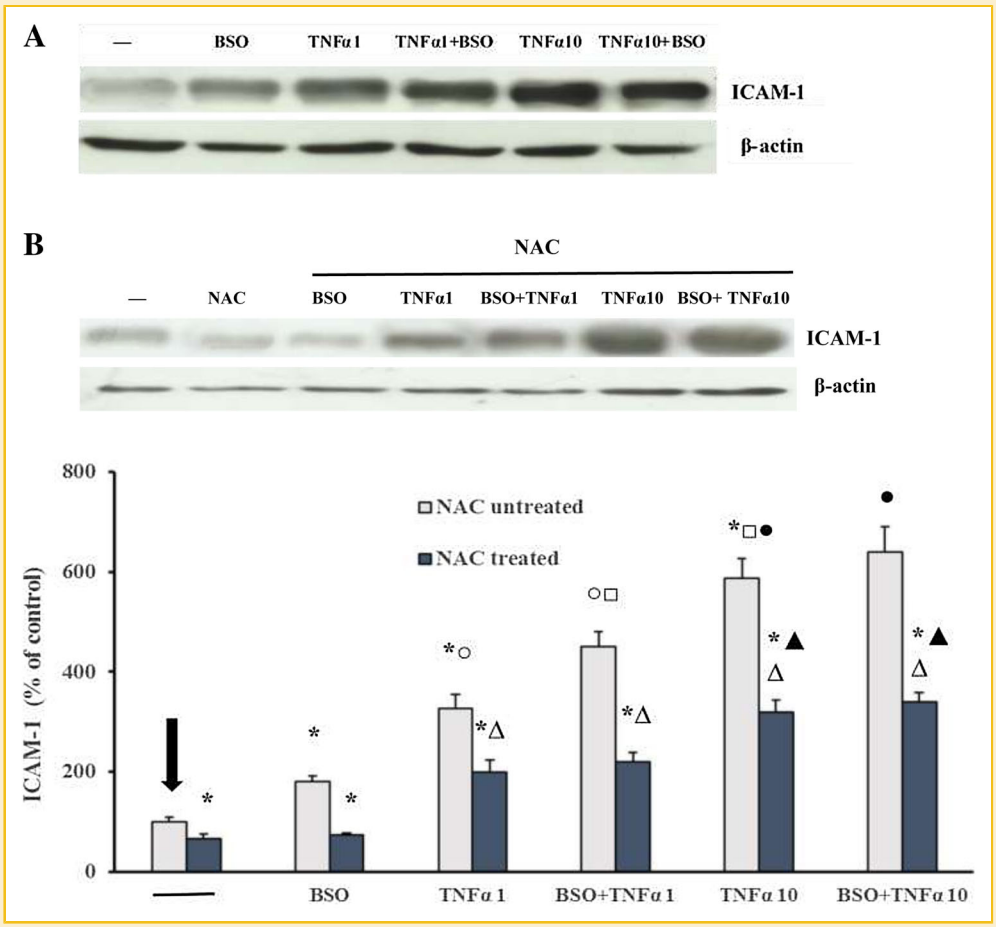

Fig. 2. ICAM-1 expression in 18Co cells treated or not with BSO or NAC and stimulated with TNFα. Starved 18Co cells, treated or not with 25 μM BSO (A) or or 20 mM NAC (B) as reported in Materials and Methods, were stimulated or not for 24 h with 1ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). For the detection of ICAM-1 expression Western blot analysis of cell lysate with anti-ICAM-1 and anti β-actin was performed. Blot is representative of four experiments and the normalized values with actin bands obtained by densitometric analysis of four experiments are reported in the bottom. The values are the mean percentage ± SEM relative to those obtained in the untreated and unstimulated cells (control, 100%, indicated with the arrow). $^{*}P \leq 0.05$ compared to the control cells. $^{\square}P \leq 0.05$ compared to NAC untreated and TNFα1 stimulated cells; $^{\circ}P \leq 0.05$ compared to NAC untreated and BSO treated cells; $^{\bullet}P \leq 0.05$ compared to NAC untreated and BSO+TNFα1 treated cells; $^{\Delta}P \leq 0.05$ compared to the respective NAC untreated cells; $^{\blacktriangle}P \leq 0.05$ compared to the respective NAC treated and TNFα1 stimulated cells.

maximum in TNFα 10 stimulated cells and significantly higher than that measured in TNFα 1 stimulated cells. Both TNFα concentrations increased significantly ICAM-1 expression as compared to BSO treated cells. ICAM-1 expression was up-regulated only in BSO + TNFα 1 treated 18Co cells, as compared to TNFα 1 stimulated cells, and the value obtained was significantly lower than that detected in 10 TNFα stimulated cells. No further increase of ICAM-1 expression was observed in BSO + TNFα 10, as compared to TNFα 10 stimulated cells (Fig. 2A). Subsequently, we measured ICAM-1 expression in 18Co cells treated with NAC. Figure 2B shows that NAC treatment in all conditions down-regulated ICAM-1 expression, but this was reduced significantly only in NAC and NAC + BSO treated cells as compared to control. In NAC treated cells stimulated with both TNFα concentrations, the expression of ICAM-1 was higher than that of control cells but significantly lower than that measured in the respective TNFα stimulated cells. Moreover, NAC significantly decreased ICAM-1 expression in both TNFα 1 and BSO + TNFα 1 cells, as compared to the value measured under the same conditions in TNFα 10 and BSO + TNFα 10 stimulated 18Co cells (Fig. 2B). In all these experimental conditions Western blot was performed on purified membranes and the results were similar to those obtained in cell lysates (data not shown). Figure 3 shows that sICAM-1 release followed roughly the same trend of ICAM-1 expression. Differently to that occurred for ICAM-1 expression, a similar release of sICAM-1 was measured in BSO treated cells and in TNFα 1 stimulated cells as compared to control value. Only TNFα 10 was able to enhance sICAM-1 release with respect to BSO treated 18Co cells. Moreover, NAC treatment restored the values of sICAM-1 release to control level in BSO treated cells and in all TNFα 1 stimulated cells but not in 18Co cells stimulated with TNFα 10. In fact, in NAC treated cells stimulated with TNFα 10 the release of sICAM-1 was higher than that measured in control cells (Fig. 3).

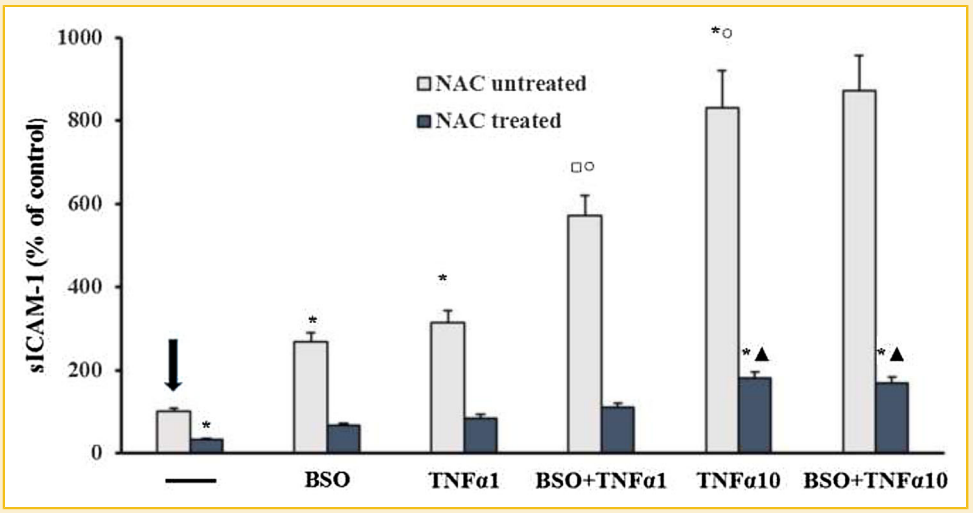

Fig. 3. sICAM-1 levels in 18Co cells treated or not with BSO or NAC and stimulated with TNFα. Starved cells, treated or not with 25 μM BSO or 20 mM NAC as reported in Materials and Methods, were stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). The culture medium was collected and used for sICAM-1 assay by immunoenzymatic method. The values, expressed as percent of untreated and unstimulated cells (control, 100%, indicated with the arrow), are the mean ± SEM of six experiments repeated in triplicate. $^{*}P \leq 0.05$ compared to the control cells. $^{\square}P \leq 0.05$ compared to NAC untreated and TNFα1 stimulated cells. $^{\circ}P \leq 0.05$ compared to NAC untreated and BSO treated cells; $^{\blacktriangle}P \leq 0.05$ compared to the respective NAC treated and TNFα1 stimulated cells.

INVOLVEMENT OF MMPs AND NADPH OXIDASE ON sICAM-1 RELEASE AND INTRACELLULAR ICAM-1 LEVELS IN 18Co CELLS TREATED WITH BSO AND STIMULATED WITH TNFα

The involvement of redox regulated MMPs on sICAM-1 release in 18Co cells treated or not with BSO and stimulated with TNFα was performed by using their inhibitor TAPI-1 [Liu et al., 2014]. Moreover, at the same time, both sICAM-1 release in culture medium and intracellular ICAM-1 levels in cell lysates were measured by ELISA kit. Data reported in Figure 4A show that BSO, TNFα, and BSO + TNFα, at both concentrations, increased intracellular ICAM-1 in agreement with the expression trend previously detected by Western blot analysis (Fig. 2A). Under these conditions TAPI-1 significantly up-regulated intracellular ICAM-1 levels (Fig. 4A) and down-regulated sICAM-1 release (Fig. 4B), as compared to the respective untreated cells. In particular, TAPI-1 treatment restored s-ICAM-1 release to control values in TNFα 1 stimulated 18Co cells treated or not with BSO, but not in all cells stimulated with TNFα 10 (Fig. 4B). Treatment of the cells with DPI, inhibitor of NADPH oxidase major producer of ROS in many cells [Panday et al., 2015], inhibited markedly both intracellular ICAM-1 and sICAM-1 release in all conditions used, as compared to the respective untreated 18Co cells (Fig. 4A, B), in accordance with the results obtained with NAC (Figs. 2B and 3). In fact, Figure 5 shows that DPI reduced also ROS production in agreement with the behavior of ROS detected in the same conditions with NAC treatment (Fig. 1).

EFFECT OF BSO, NAC, DPI, AND TNFα ON TACE EXPRESSION AND ACTIVATION IN 18Co CELLS

TACE expression and activation, detected by Western blot assay, were measured by densitometric analysis of pro-TACE at 120 kDa and mature (active) TACE at 80 kDa bands, respectively. These enhanced both in BSO treated and in TNFα stimulated cells, as compared to the respective controls (Fig. 6A). Both expression and activation of TACE significantly increased in TNFα 1 + BSO cells, when compared with 18Co BSO treated or TNFα 1 stimulated cells, reaching the values measured in all cells stimulated with TNFα 10. Thr735 phosphorylation of TACE cytoplasmic tail is very important for TACE mobilization to plasma membrane and activation [Scheller et al., 2011]. The use of a specific antibody, which detects endogenous levels of TACE phosphorylation only when phosphorylated at Thr735, shows that the trend of this phosphorylation, relative to 80 kDa band, was similar to that of TACE activation in all conditions used (Fig. 6A). However, the proportion of TACE phosphorylated, calculated by ratio between bands of TACE P-Thr735 and those of pro-TACE (120 kDa), was similar in all conditions (Fig. 6B). Moreover, Figure 6B shows also that mature TACE was all phosphorylated, as indicated by ratio between bands of TACE P-Thr735 and those of TACE at 80 kDa. Treatment with NAC and DPI restored TACE expression, activation and phosphorylation to values of control in all conditions (Fig. 7A, B).

DISCUSSION

In the present study, we examined the role of intracellular oxidative stress on ICAM-1 expression and sICAM-1 release in 18Co cells stimulated or not with TNFα. In particular, we demonstrated, for the first time, an increase of ICAM-1 expression and sICAM-1 release in condition of oxidative stress in 18Co cells. The involvement of redox regulated MMPs in the up-regulation of sICAM-1 release has been also demonstrated. Moreover, this study shows ROS dependent increase of TACE activation and expression suggesting its possible

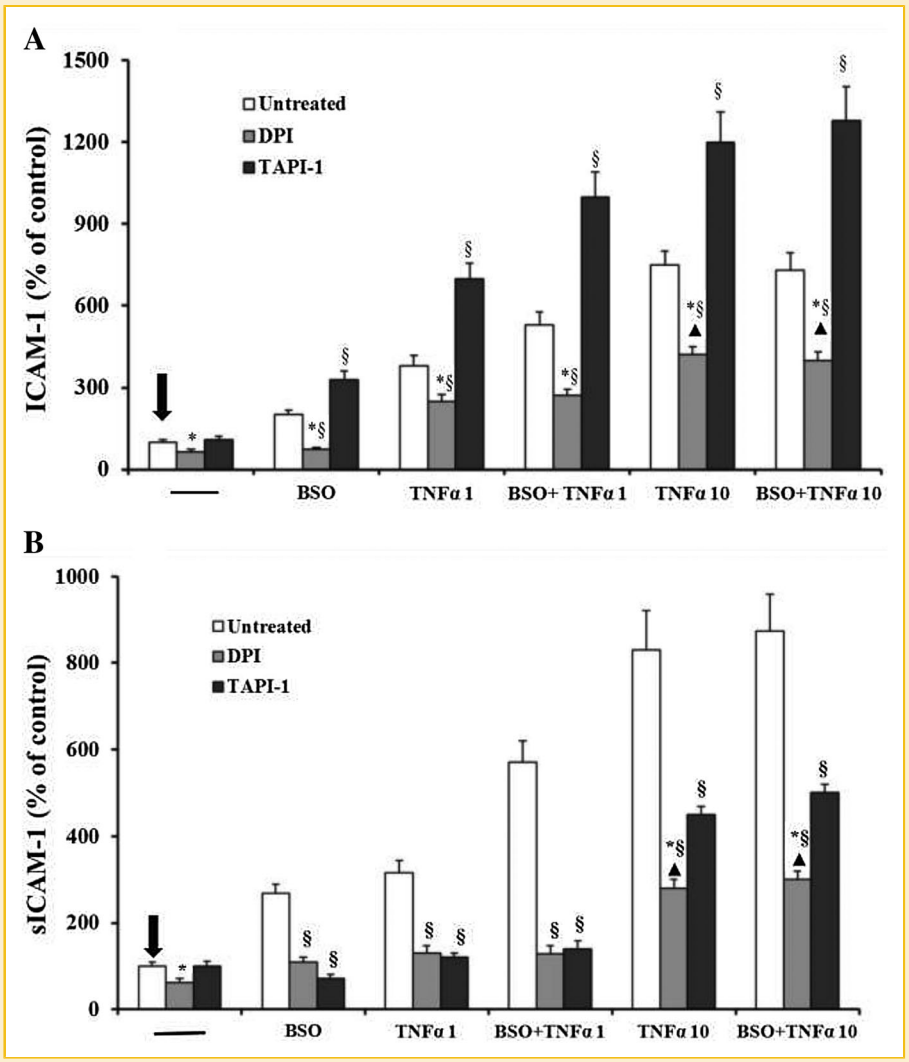

Fig. 4. Effect of TAPI-1 and DPI on intracellular ICAM-1 levels and sICAM-1 levels in 18Co cells treated or not with BSO and stimulated with TNFα. 10 μM TAPI-1 or 10 μM DPI were added to starved cells treated or not with 25 μM BSO and stimulated or not for 24 h with 1 ng/ml TNFα (TNFα 1) or 10 ng/ml TNFα (TNFα10), as reported in Materials and Methods. Intracellular ICAM-1 levels (A) and sICAM-1 (B) assays were performed by ELISA kit in cell lysates and in culture medium, respectively. The values, expressed as percent of untreated and unstimulated cells (control, 100%, indicated with the arrow), are the mean ± SEM of six experiments repeated in triplicate. $^{*}P \leq 0.05$ compared to the control cells; $^{\S}P \leq 0.05$ compared to the respective untreated cells; $^{\blacktriangle}P \leq 0.05$ compared to the respective TNFα 1 stimulated cells.

role in the up-regulation of sICAM-1 release. In particular, BSO treatment or TNFα stimulation of 18Co cells induce a rise of ROS production related to an increase of ICAM-1 expression and sICAM-1 release. In fact, NAC and DPI treatments reduce or restore ROS production to control and down-regulate ICAM-1 expression and release. TNFα causes oxidative stress [Woo et al., 2000; Lee et al., 2013] and induces ROS production in a concentration-dependent manner. However, in BSO treated cells, only the physiological concentration of TNFα (1 ng/ml) is able to increase ROS production reaching the value obtained in cells stimulated with 10 ng/ml TNFα. This suggests that TNFα at physiological concentrations, in the presence of intracellular oxidative stress, increases this inducing tissue damage as it occurs in pathological concentrations. This is particularly important in pathologies, as IBD, in which a condition of oxidative stress is present [Iantomasi et al., 1994; Buffinton and Doe, 1995; O'Connor et al., 2010; Catarzi et al., 2011a]. It is to note that ROS production is similar in BSO treated or TNFα 1 stimulated cells whereas ICAM-1 expression is different. In fact, ICAM-1 expression is higher in TNFα 1 stimulated cells than in cells treated with BSO. NAC treatment down-regulates ICAM-1 expression only in control and in BSO treated 18Co cells in according to ROS levels. NAC lowers the expression of ICAM-1 levels, but is not able to restore them to the control values in cells stimulated with both concentrations of TNFα, differently to that occurs for ROS production. These results indicate that the up-regulation of ICAM-1 expression in TNFα stimulated 18Co cells, is partially dependent on ROS production and is related to TNFα concentrations through a ROS-independent mechanism. Oxidative stress induces ICAM-1 expression [Roebuck et al.,

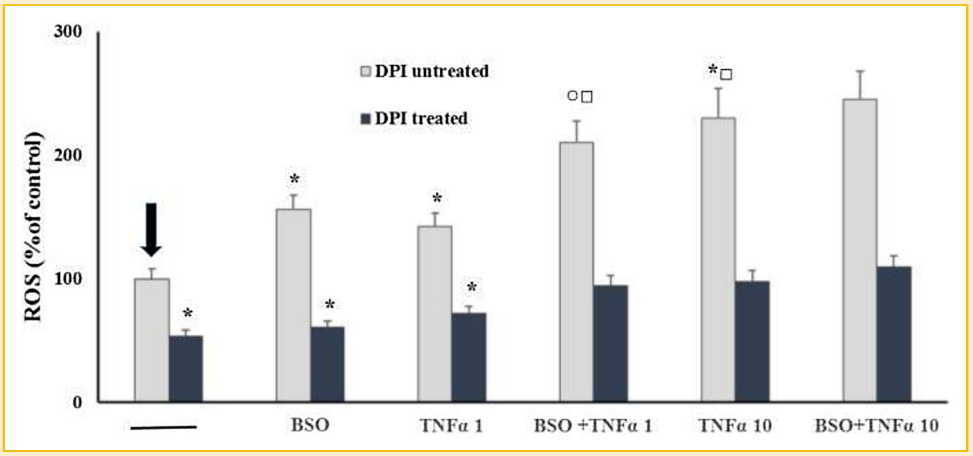

Fig. 5. Effect of DPI on intracellular ROS production in 18Co cells treated with BSO and stimulated with TNFα. Starved 18Co cells, treated or not with 25 μM BSO in the presence of 10 μM DPI as reported in Materials and Methods, were stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). 0.003% DMSO (vehicle for DPI) was added to DPI untreated cells. The intracellular ROS production was detected by measuring the fluorescence intensity of the intracellular oxidation-sensitive probe H_2DCFDA. The values, normalized on total protein content and expressed as percent of untreated and unstimulated cells (control, 100%, indicated with the arrow), are the mean ± SEM of six experiments repeated in triplicate. $^{*}P \leq 0.05$ compared to the control cells; $^{\square}P \leq 0.005$ compared to DPI untreated and TNFα1 stimulated cells; $^{\circ}P \leq 0.001$ compared to DPI untreated and BSO treated cells.

1995] whose redox regulation occurs at different levels being cell type- and stimulus-dependent [Roebuck and Finnegan, 1999]. In lung epithelial cells, TNFα-induced ICAM-1 increase is due to the activation of redox regulated signaling pathways, and in fact, it is inhibited by pre-treatment with NADPH-oxidase inhibitor or antioxidants [Kim et al., 2008; Lee et al., 2013; Yen et al., 2013]. However, in endothelial cells TNFα stimulation of ICAM-1 is different from that induced by exogenous H_2O_2 [Roebuck and Finnegan, 1999], and the down-regulation of TNFα-induced myogenesis occurs in murine skeletal muscle cells through redox-dependent and -independent pathways [Langen et al., 2002]. The treatment of 18Co cells with DPI demonstrates the possible involvement of NADPH oxidase in the increase of ICAM-1 levels; in fact this enzyme represents the principal source of ROS induced by TNFα [Li et al., 2005]. However, DPI, similarly to NAC, reduces ROS levels, but does not remove totally the effect of TNFα on ICAM-1 expression. This confirms also the involvement of a redox-independent regulation in the increase of TNFα-induced ICAM-1 expression in 18Co cells.

A closer relationship is demonstrated between sICAM-1 release and the oxidative stress. This is especially evident in 18Co cells treated with BSO or stimulated with TNFα 1, also in the presence of NAC and DPI treatments. On the contrary, sICAM-1 release in TNFα 10 stimulated cells is only in part related to ROS production, given that NAC and DPI do not restore sICAM-1 levels to those of control, differently to that occurs for ROS levels. Therefore, we suggest that TNFα at high concentrations up-regulates sICAM-1 release also by activating other redox-independent mechanisms responsible for proteolytic cleavage of cell surface ICAM-1. sICAM-1 release was significantly inhibited by TAPI-1, indicating that redox regulated MMPs are involved in the cleavage of transmembrane ICAM-1 induced by oxidative stress in 18Co cells. Alterations of the cellular redox state may regulate both activation and expression of TACE, a protease that cleaves membrane-bound proteins [Scheller et al., 2011]. However, in literature no data are reported on the expression, activation and regulation of this enzyme in ISEMFs in the presence or not of changes in intracellular oxidative state. For the first time, in 18Co cells an up-regulation of the expression and activation of TACE, related to intracellular oxidative stress, has been demonstrated. In fact, TACE expression and activation correlate with ROS levels in cells treated with BSO and/or stimulated with TNFα. Moreover, NAC and DPI treatments restore them to control values in all conditions. Given this and considering that some authors indicate TAPI-1 as specific inhibitor of TACE [Liu et al., 2014], a possible role of TACE in the cleavage of ICAM-1 in the presence of oxidative stress cannot be excluded. The simultaneous enhancement of endogenous ICAM-1 detected in TAPI-1 treated cells can be due to the inhibition of ICAM-1 shedding. Moreover, it is also possible that, in TNFα treated cells, TAPI-1 blocks TACE-mediated generation of antagonistic soluble TNFα receptors [Scheller et al., 2011]. This can increase ICAM-1 expression trough an enhanced cell stimulation by TNFα. Exogenous H_2O_2 activates TACE, involving the cysteinyl sulphydryl groups in the extracellular domain [Wang et al., 2009], and increases its cell surface expression via p38-mediated TACE phosphorylation [Brill et al., 2009]. In fact, the activation of this MAPK leads to specific phosphorylation of Thr735 involved in the TACE translocation to membrane and activation. In our conditions, the trend of Thr735 phosphorylation relative to 80 kDa band agrees with TACE activation and it is related to ROS levels. The increased phosphorylation of TACE correlates with the up-regulation of its expression. In fact, the absolute values of TACE phosphorylation change, whereas the proportion of phosphorylated TACE is similar in all conditions. We suggest the involvement of p38 MAPK on TACE phophorylation, considering that a redox-regulated activation of this MAPK occurs in 18Co cells stimulated or not with TNFα and in ISEMFs isolated from intestine of CD patients [Catarzi et al., 2011a]. Therefore, the redox

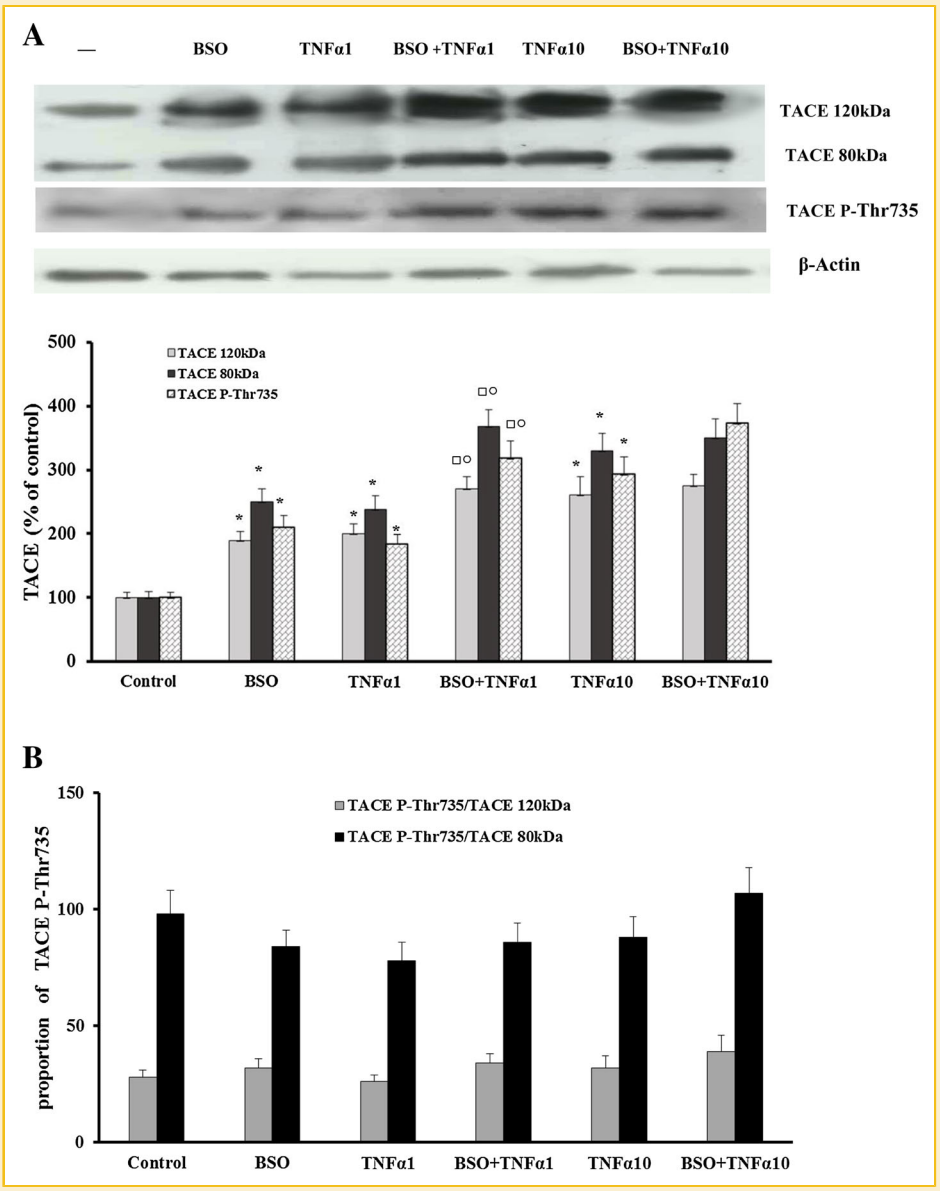

Fig. 6. TACE expression and activation in 18Co cells treated or not with BSO and stimulated with TNFα. Starved cells, treated or not with 25 μM BSO as reported in Materials and Methods, were stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10). Western blot analysis of cell lysate with anti-TACE or anti-ADAM17-phosphoThr735 or anti β-actin was performed for the detection of TACE expression and activation. The TACE P-Thr735 was detected on 80 kDa band. A: Blots are representative of four experiments and normalized values with actin band obtained by densitometric analysis of four experiments are reported in the bottom. The values are the mean percentage ± SEM relative to those obtained in the untreated and unstimulated cells (control, 100%). B: The proportion of TACE phosphorylated was calculated both by ratio between bands of TACE P-Thr735 and those of pro-TACE (120 kDa) and by ratio between bands of TACE P-Thr735 and those of mature TACE (80 kDa). $^{*}P \leq 0.005$ compared to the control cells. $^{\square}P \leq 0.05$ compared to the respective TNFα1 stimulated cells $^{\circ}P \leq 0.05$ compared to the respective BSO treated cells.

regulation of TACE expression and activation, detected in intestinal myofibroblasts by us, may be involved in the pro-inflammatory activity of TNFα in inflamed gut. In fact, myofibroblasts produce TNFα in intestinal inflammatory state and, in CD patients, they show high expression of transmembrane TNFα [Di Sabatino et al., 2007]. Moreover, TACE is required in cell surface proteolytic processing of TNFα that after cleavage assumes its inflammatory properties [Scheller et al., 2011].

ICAM-1 is involved in the recruitment of immune cells at inflammatory sites, and the infiltration and migration of leukocytes into inflamed intestinal mucosa and submucosal layers are crucial for the pathogenesis of IBD [Binion et al., 2009]. ISEMFs produce molecules that contribute to this migration [Pinchuk et al., 2010], therefore the up-regulation of ICAM-1 induced by oxidative stress and TNFα stimulation in ISEMFs can be involved in the excessive migration of leukocytes to the site of local gastrointestinal mucosal

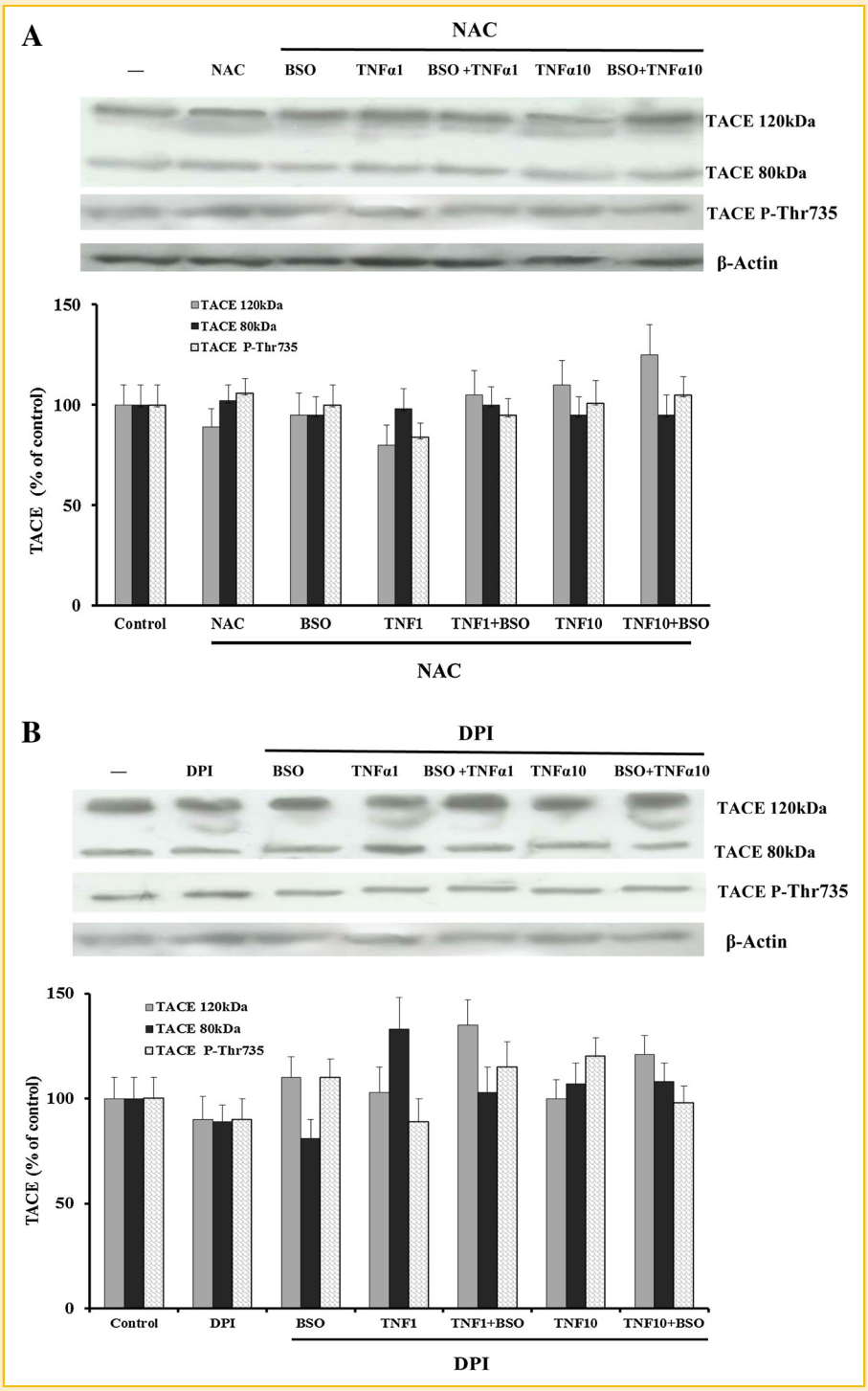

Fig. 7. Effect of NAC or DPI on TACE expression and activation in 18Co cells treated or not with BSO and stimulated with TNFα. 20 mM NAC or 10 μM DPI were added to starved cells treated or not with 25 μM BSO and stimulated or not for 24 h with 1 ng/ml TNFα (TNFα1) or 10 ng/ml TNFα (TNFα10), as reported in Materials and Methods. 0.003% DMSO (vehicle for DPI) was added to DPI untreated cells. Western blot analysis of cell lysate with anti-TACE or anti-ADAM17-phosphoThr735 or anti β-actin was performed for the detection of TACE expression and activation. The TACE P-Thr735 was detected on 80 kDa band. Blots are representative of four experiments and normalized values with actin band obtained by densitometric analysis of four experiments are reported in the bottom. The values are the mean percentage ± SEM relative to those obtained in the untreated and unstimulated cells (control, 100%).

inflammation. Then, we speculate that this could be in part responsible of the excessive recruitment of leukocytes in CD patient intestine. In fact, an oxidative state condition is present also in ISEMFs [Iantomasi et al., 1994; Catarzi et al., 2011a] as well as an increase of ICAM-1 and sICAM-1 in CD intestinal tissue [Ghosh and Panaccione, 2010]. The increase of sICAM-1 levels in conditions of oxidative stress and/or TNFα stimulation suggests also that ISEMFs can contribute in the enhancement of sICAM-1 levels in serum of IBD patients. In fact, it has been demonstrated that the increase of sICAM-1 in the blood of these patients is related to the levels present in intestinal mucosa and derives from activated cells within gut mucosa and not from circulating leukocytes [Nielsen et al., 1994; Song et al., 2009]. NAC, down-regulating ICAM-1 expression and sICAM-1 release in ISEMFs, can reduce the excessive recruitment of immune cells in intestinal mucosa, the activation of inflammatory cascade and angiogenesis, preventing the onset and the progression of chronic inflammatory diseases [Lawson and Wolf, 2009; Angel-Morales et al., 2012]. Moreover, the reduction of ICAM-1expression and of its soluble form by antioxidants treatment could interfere with the tumor differentiation and the metastasis development. In fact, intestinal myofibroblasts regulate the migration, differentiation and growth of normal and carcinoma cells [Pinchuk et al., 2010], and the chronic inflammation can favor the development of colon cancer [Grivennikov, 2013]. Since our results show that in TNFα stimulated 18Co cells NAC treatment reduces in part ICAM-1 expression and sICAM-1 release, we speculate that the synergistic effect between the antioxidants and anti- TNFα drugs could be therapeutically useful.

CONCLUSIONS

All together, our data demonstrate, for the first time in 18Co cells, an up-regulation of ICAM-1 expression and sICAM-1 release induced by oxidative stress and TNFα stimulation, showing a redox regulation of these events. However, also redox-independent mechanisms in ICAM-1 expression and release are probable in TNFα stimulated cells. It was shown that cleavage of ICAM-1 on cell surface is mediated by redox regulated MMPs. Moreover, we suggest the possible involvement of TACE, whose expression and activation in 18Co cells have been related to ROS levels. An involvement of NADPH oxidase in the redox regulation of ICAM-1 and TACE has been demonstrated.

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Oxidative stress in bone remodeling: role of antioxidants

Vladana Domazetovic[1]
Gemma Marcucci[2]
Teresa Iantomasi[1]
Maria Luisa Brandi[2]
Maria Teresa Vincenzini[1]

[1] Department of Biomedical, Experimental and Clinical Sciences "Mario Serio" (Biochemistry section), University of Florence, Florence, Italy
[2] Department of Surgery and Translational Medicine (Endocrinology Section), University of Florence, Florence, Italy

Address for correspondence:
Maria Teresa Vincenzini
Department of Biomedical, Experimental and Clinical Sciences
University of Florence
Viale Morgagni 50
50134 Florence, Italy
Phone: +39-055-2751238; Fax: +39-055-7830303
E-mail: vincenzini@unifi.it

Summary

ROS are highly reactive molecules which consist of a number of diverse chemical species, including radical and non-radical oxygen species. Oxidative stress occurs as a result of an overproduction of ROS not balanced by an adequate level of antioxidants. The natural antioxidants are: thiol compounds among which GSH is the most representative, and non-thiol compounds such as polyphenols, vitamins and also various enzymes.
Many diseases have been linked to oxidative stress including bone diseases among which one of the most important is the osteoporosis. The redox state changes are also related to the bone remodeling process which allows the continuous bone regeneration through the coordinated action of bone cells: osteoclasts, osteoblasts and osteocytes. Changes in ROS and/or antioxidant systems seem to be involved in the pathogenesis of bone loss. ROS induce the apoptosis of osteoblasts and osteocytes, and this favours osteoclastogenesis and inhibits the mineralization and osteogenesis. Excessive osteocyte apoptosis correlates with oxidative stress causing an imbalance in favor of osteoclastogenesis which leads to increased turnover of bone remodeling and bone loss. Antioxidants either directly or by counteracting the action of oxidants contribute to activate the differentiation of osteoblasts, mineralization process and the reduction of osteoclast activity. In fact, a marked decrease in plasma antioxidants was found in aged or osteoporotic women. Some evidence shows a link among nutrients, antioxidant intake and bone health. Recent data demonstrate the antioxidant properties of various nutrients and their influence on bone metabolism. Polyphenols and anthocyanins are the most abundant antioxidants in the diet, and nutritional approaches to antioxidant strategies, in animals or selected groups of patients with osteoporosis or inflammatory bone diseases, suggest the antioxidant use in anti-resorptive therapies for the treatment and prevention of bone loss.

KEY WORDS: oxidative stress; antioxidants; bone remodeling; osteoporosis; antioxidant nutrient treatment.

Introduction

The physiological intracellular redox state depends on the ratio between the levels of pro-oxidants, oxidizing agents (reactive oxygen species, ROS) and antioxidants (1, 2). ROS are highly reactive molecules, which consist of a number of diverse chemical species, including radical and non-radical oxygen species such as superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). O_2^-, considered as the 'primary' ROS, can further interact with other molecules in order to generate 'secondary' ROS which are more aggressive, and they act either directly or prevalently through enzyme or metal-catalysed processes. ROS are produced during normal metabolism following the activation of various enzymes such as NADPH oxidase (membrane enzyme), superoxide dismutase (cytoplasmic enzyme) and various mitochondrial oxidases (3, 4). Indeed, a controlled increase of ROS level and in particular of H_2O_2 may have an important role in the transmission of intracellular signaling which regulate many fundamental cellular processes such as proliferation, differentiation, apoptosis, repair processes and inflammation (5, 6).
The natural antioxidants are: thiol compounds among which the most important and represented in animals is the glutathione (GSH, γ-glutamyl-cysteinyl-glycine), non-thiol compounds such as polyphenols, predominantly contained in various plants, vitamins such as ascorbic acid, alfa-tocopherol and vitamin A, as well as various enzymes capable to eliminate ROS such as catalase, and enzymes that use GSH as substrate (GSH-reductase, GSH-peroxidase etc.) (3). GSH, present in concentrations of 2-10 mM within cells, is the primary determinant of the cellular redox environment (7) and exists mainly as the biologically active reduced-thiol form. The oxidation of GSH to GSSG and subsequent decrease in the GSH/GSSG ratio is often associated with oxidative stress. Thus, the GSH/GSSG ratio is a simple and useful indicator of cellular redox state (8, 9). *De novo* GSH synthesis, GSSG reduction, and exogenous GSH uptake are crucial in the maintenance of cellular redox homeostasis, and GSH seems to be involved in signaling

pathways being able to regulate the activity of transcription factors and protein through reactions of glutathionilation (2, 10, 11). Other thiol antioxidants are: Thioredoxin, Glutaredoxin, Cysteine (Cys) and the reduced form of Lipoic Acid (LA), dihydrolipoic acid (DHLA): the first two have catalitic-redox-active cysteines and catalize the reduction of protein mixed disulfides (3). Cys is the most abundant low-molecular thiols in extracellular fluids with concentrations ranging from 40 μM to 8-10 μM (12), and DHLA contains two thiol groups and is produced in almost every cell in small amounts. *In vivo* Cys and DHLA act directly on ROS and RNS (Reactive Nitrogen Species) as scavengers and by regeneration of other antioxidants such as vitamin C, E and GSH (13-15).

The physiological redox state is maintained in equilibrium by various factors and mechanisms that regulate the activity of ROS-producing enzymes and antioxidants. Oxidative stress occurs as a result of an overproduction of ROS not balanced by adequate levels of antioxidants (1, 2). This can be determined by both physiological events, such as aging and hormonal changes (decrease of estrogen) (16-19) and pathological events related to the production of inflammatory cytokines involved in many pathological processes, exogenous and endogenous toxins, radiation exposure and drug therapies (2, 6, 20).

Oxidative stress generates a cellular damage due to lipid oxidation, structural alteration of the membranes, oxidation of proteins and nucleic acids; the damage may extend to the organs and become systemic (21). Many diseases have been linked to oxidative stress including bone diseases among which one of the most important is the osteoporosis. Oxidative stress in postmenopausal osteoporosis, due to estrogen deficiency, has been related to the activation of NADPH oxidase and/or decreased synthesis of antioxidant enzymes and GSH levels (17, 18, 22, 23). Oxidative stress, in the elderly osteoporosis and in the secondary osteoporosis due to intestinal chronic diseases (IBD), is due to decrease of GSH levels and defensive antioxidant abilities (19, 20, 24) related also to reduced intestinal absorption of antioxidants contained in food. In osteoporosis secondary to bone inflammatory processes and prolonged therapy with steroidal anti-inflammatory drugs, oxidative stress is mainly due to the activation of enzymes which produce ROS (25, 26).

Oxidative stress in bone remodeling

The redox state changes are also related to the bone remodeling process which allows the continuous bone regeneration (25, 27). In fact, bone is a dynamic tissue that continuously renews itself throughout life by the coordinated action of three major types of bone cells: osteoclasts, osteoblasts and osteocytes (28, 29). The remodeling process is the result of interactions between these cells and multiple molecular agents, including hormones, growth factors and cytokines. It is a physiological process that follows a time sequence lasting approximately six months wherein osteoclasts eliminate old or damaged bone tissue which is subsequently replaced with new bone tissue formed by osteoblasts, while the osteocytes function in the transduction of signals necessary to sustain mechanical loads. Recently, new data support the central regulatory role of osteocytes in bone remodeling and thus in viability and functionality of bone, maintaining normal levels of mineralization and repairing microdamage and microfractures (30-32). Healthy bone is tightly regulated and maintained in order to prevent significant alterations in bone mass or mechanical strength after each remodeling cycle (29).

Indeed, oxidative stress alters bone remodeling process causing an unbalance between osteoclast and osteoblast activity, this can lead to metabolic bone diseases and contribute to the pathogenesis of skeletal system disorders including osteoporosis characterized by low bone mineral density and decrease in bone mass, which makes the bone weak and more prone to fracture (18, 33-36). Recent evidences in a limited number of clinical studies have shown that ROS and/or antioxidant systems can be involved in the pathogenesis of bone loss (36-39).

In fact, oxidative stress activates the differentiation of pre-osteoclasts in osteoclasts and strengthens the bone resorption (18, 40) (Figure 1). A significant increase in the number and activity of osteoclasts as well as in the tartrate-resistant acid phosphatase level was observed when H_2O_2 was added to cultures of human marrow mononuclear cells (36).

ROS induce the apoptosis of osteoblasts and osteocytes, cells localized in the bone matrix and derived from mature osteoblasts, thus favouring osteoclastogenesis (19, 30, 31, 41, 42) (Figure 1). In fact, ROS elicit a spectrum of responses ranging from proliferation, growth, differentiation arrest to cell death, by activating numerous signalling pathways. Indeed, mitogen-activated protein kinase (MAPKs) such as extracellular signal-regulated kinases (ERK1/2), c-Jun-N terminal kinase (JNK) and p38 MAPK are involved in osteoblast or osteocytes apoptosis (15, 43, 44). High levels of ROS block and reduce the osteoblast activity and differentiation, therefore the mineralization and osteogenesis (8, 45, 46) (Figure 1). These events increase bone remodeling turnover with consequent alteration and decrease in bone mass. Antioxidants have opposing effects, they contribute to the differentiation of osteoblasts and bone formation (8, 15, 34, 47), maintaining vital osteocytes which contribute to osteoblast activity and osteogenesis, while reduce the osteoclast differentiation and their activity (Figure 1).

There are several factors mainly produced by osteoblasts and osteocytes that regulate osteoclast and osteoblast activity and then bone remodeling, among these the most important are: the ligand of receptor activator of NF-kB (RANKL) and osteoprotegerin (OPG). Their expression is sensitive to increased oxidative status, that induces RANKL up-regulation and OPG down-regulation through the activation of protein kinases (ERK1/2, JNK etc.) and/or other factors which affect specific transcription factors (8, 15, 27). RANKL activates the differentiation and activity of osteoclasts by interacting with specific receptors in pre-ostoeclasts and mediates osteoclastogenesis and bone resorption; while OPG, produced by the activation of the signaling pathway Wnt/βcatenin, is a soluble receptor capable of binding and blocking RANKL, resulting in inhibition of osteoclast activity (30-32, 42, 48) The oxidative stress blocks the activation of osteoblasts and thus the production of OPG; under this condition, the action of RANKL prevails, and the differentiation and activity of osteoclasts are induced. Thereafter, the turnover of the bone remodeling process increases and this is indicated by an increase in RANKL / OPG ratio, that is, in fact, an index of the intensity of bone resorption (18, 49). The regulation of the RANKL/OPG ratio levels is responsible for the

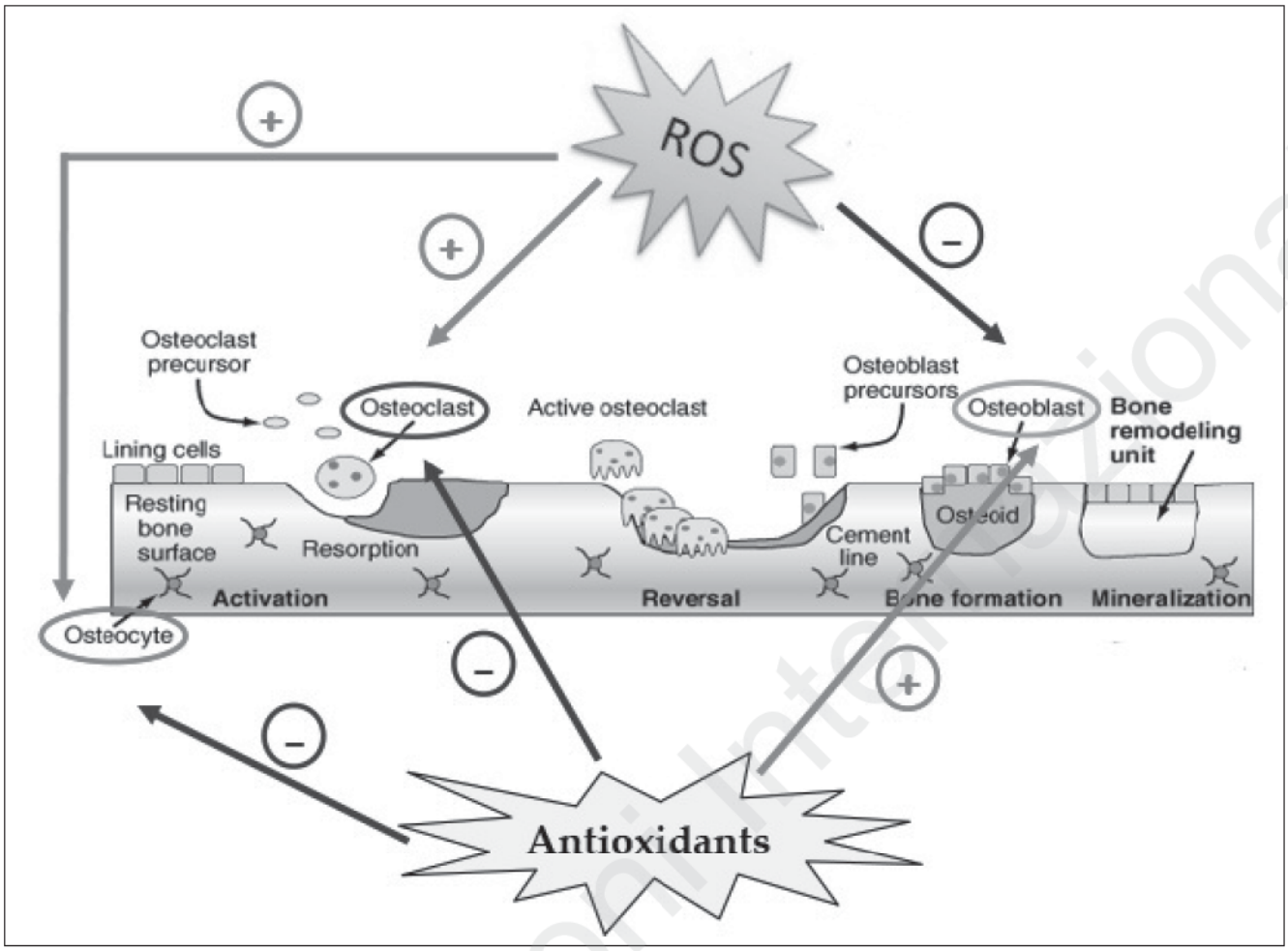

Figure 1 - Effect of ROS and antioxidants on the activity of osteoclasts, osteoblasts and osteocytes in bone remodeling. ROS activate osteoclast differentiation and osteocyte apoptosis (+), while inhibit osteoblast activity (-) inducing bone resorption; antioxidants activate osteoblast differentiation (+) and inhibit osteoclast activity and osteocyte apoptosis (-) inducing bone formation.

maintenance of balance between bone resorption and formation. Increased levels of this ratio are correlated to increased turnover of bone remodeling due to increased resorption rate without adequate and proper bone formation; this event has been related to the pathogenesis of various skeletal diseases, including various form of osteoporosis and bone diseases secondary to inflammation (31, 32, 48). The expression of RANKL and OPG is regulated by various hormones and cytokines, but a fundamental event in the initiation and regulation of the bone formation and remodeling is the apoptosis of osteoblasts and osteocytes (27, 32).

Experimental data show that excessive apoptosis of osteocytes is correlated to an increased oxidative status causing an imbalance in favor of osteoclastogenesis (15, 50-52). There are few data on the molecular mechanisms that regulate these processes, but many studies are currently focused on the regulatory activities of the osteocytes. These cells constitute 90% of the bone cell population and are embedded in the bone matrix. They have a morphology similar to neuronal cells with a central body and dendritic extensions thanks to which they communicate with each other, with other bone cells, with blood capillaries and nerve endings. They are mechanosensory cells (30, 41) and under physiological conditions, following a microdamage or other physical and hormonal signals, such as estrogen deficiency, mature osteocytes undergo apoptosis, and some data show that this is related to oxidative stress (42, 50, 51). Indeed, apoptotic osteocytes induce ligning cells to retract from the bone surface to form a suitable environment for the recruitment and activation of mature osteoclasts through formation and release of RANKL (32, 53). They also produce high levels of sclerostin and DKK1 which block OPG synthesis and release by Wnt/βcatenin pathway inhibition both in osteocytes and osteoblasts (30-32, 42). This event increases RANKL/OPG ratio promoting osteoclast activity, osteoblast apoptosis and bone degradation. Indeed, a microfracture can determine the breaking of the dendritic filaments and therefore the connections with other cells and blood vessels. This causes a deficient intake of O_2, nutrients, hormones and factors essential for their viability inducing metabolic alterations, oxidative stress and osteocyte apoptosis which initiates the remodeling process and bone resorption (34, 41, 42, 52, 54).

Under physiological conditions, after the phase of bone resorption, in response to factors released from the bone matrix, the recruitment of osteoblast precursors and their differentiation into mature, bone-synthesizing cells occur. However, an excessive oxidative stress induces an abnormal apoptosis of osteocytes resulting in an imbalance of the

remodeling process with consequent altered and deficient bone formation, as occurs in aging, glucocorticoid treatment, osteoporosis and other bone diseases related to oxidative stress (17, 26, 31, 55-57). Differently, vital osteocytes produce high levels of OPG and this contributes to the differentiation of osteoblasts and mineralization process (32, 50).

Antioxidants in bone remodeling and in bone loss

In vivo and *in vitro* data have shown that thiol and non-thiol antioxidants, directly and/or counteracting the effect of oxidants contribute to activation of osteoblast differentiation, mineralization process and reduction of osteoclast activity. All these antioxidants act as direct scavengers of ROS, but they also maintain high levels of GSH, in fact, together with GSH-reductase they contribute to the elimination of GSSG formed in the reduction reactions, maintaining normal levels of GSH/GSSG and the intracellular redox state (8, 14, 47). Some studies relate antioxidants to bone metabolism, in fact a marked decrease in plasma antioxidants was found in aged or osteoporotic rats and in aged or osteoporotic women (17, 19, 38). The loss of antioxidant leads to accelerated bone loss through the activation of a tumour necrosis factor alpha (TNFα)-dependent signalling pathway (18), and the administration of antioxidants such as vitamin C, E, N-acetylcysteine (NAC) and LA, has beneficial effects in individuals with osteoporosis (56-59). Administration of NAC or ascorbate in ovariectomized mice abolishes ovariectomy-induced bone loss, while l-buthionine-(S,R)-sulphoximine (BSO), a specific inhibitor of glutathione synthesis, causes substantial bone loss (49). LA has also beneficial effects in the maintenance of a healthy bone structure in rat ovariectomy and inflammation-mediated osteoporosis (60). Other data demonstrate that the administration of vitamin E is able to maintain bone mineral density in elderly men (39), and it promotes healing of osteoporotic fracture in ovariectomized rats inducing the bone regeneration (61).

However, few studies have been performed on the direct action of antioxidants on bone cell activity. As regards thiol antioxidants some data have been obtained by using NAC, GSH and LA. NAC, a cysteine analogue drug with many therapeutic applications, has a protective role in controlling oxidative stress against many cells including osteoblasts, and stimulates osteoblastic differentiation of mouse calvarial cells (8, 14, 47, 62). Other studies report that NAC inhibits oxidative stress induced apoptosis of osteoblastic cells, and this is mediated by GSH (63, 64), moreover, NAC prevents osteoclast formation, NF-κB activation and TNF-α expression involved in osteoclast activation (49). Indeed, GSH is involved in osteoblast and osteoclast differentiation as well as in osteoporosis and other bone diseases (14, 17, 65).

Data reported in human osteoblastic-like SaOS-2 cells, which represent a valuable model system for studying osteoblast functions and mineralization process, demonstrate that GSH and NAC increase alkaline phosphatase (ALP) activity and other osteogenic markers, such as RUNX-2 and osteocalcin, during the differentiation of these cells in mature osteoblasts (8). These antioxidants are able to restore osteoblastic mineralization due to oxidative stress induced both by GSH depletion, obtained by BSO (8) and by H_2O_2 treatment in bone marrow stromal cells isolated from rat femur (62). LA is also able to inhibit TNFα induced apoptosis of human bone marrow stromal cells in which JNK and NFkB are involved (66). It has also been demonstrated that high levels of GSH/GSSG ratio are important for osteoblast differentiation and mineralization process (8). Indeed, GSH redox state has been reported to play an important role in the differentiation and phenotype expression of some cell types including osteoblasts and osteoclasts (67). Moreover, GSH and NAC in SaOS-2 cells decrease RANKL/OPG levels, while, increase calcium levels and mineralization process (8). Therefore, these antioxidants promote bone formation and have an anti-osteoclastogenic action. LA also suppresses osteoclastogenesis in hBMSC by directly inhibiting RANKL-RANK mediated signals (13). Recent data have demonstrated the ability of GSH, NAC and LA to prevent osteocyte apoptosis and the increase of sclerostin and RANKL/OPG ratio levels induced by oxidative stress (15). This study is performed in a murine osteocyte-like cell line, MLO-Y4, which has similar phenotype and many characteristics of mature osteocytes and constitute a model to study osteocyte viability and apoptosis in response to microdamage and bone diseases (41, 52). This study was accomplished by inducing oxidative stress via starvation that, in part, mimics what happens following a microdamage at the level of osteocytes (34, 41, 42, 52, 54). Antioxidants and ROS mediate starvation-induced apoptosis and OPG expression by JNK signalling; whereas they regulate RANKL and sclerostin expression by both JNK and ERK1/2 activity (15). Moreover, it has been demonstrated that catalase, used as antioxidant, is able to inhibit the activation of TRAP-positive multinucleated giant cells induced by H_2O_2 treatment in cultures of primary human marrow cells (36). Other antioxidants inhibit TRAP, k-catepsin and protease activity in osteoclast cell lines (68, 69). In Figure 2 the effects of antioxidants against the action of oxidative stress at the molecular and cellular level are summarized. At the molecular level they prevent RANKL and sclerostin increase and OPG decrease, inhibiting the enhancement of the RANKL / OPG ratio in osteoblasts and osteocytes. Moreover, they inhibit the increase of bone acid phosphatase and protease activity which degrade the bone matrix in osteoclasts and induce alkaline phosphatase and matrix protein synthesis in osteoblasts. At the cellular level they counteract the excessive apoptosis of osteoblasts and osteocytes and reduce the differentiation and activity of osteoclasts.

Role of nutrients containing antioxidants in treatment and prevention of bone loss

Given the important role of ROS and oxidative stress in bone turnover, there is a considerable interest in the use of antioxidants in potential treatments for osteoporosis and bone inflammatory diseases. Different experimental protocols have been studied using either pharmacological or nutritional approaches. As regards nutritional approaches, epidemiological studies have provided evidence of a link between nutrient, antioxidant intake and bone health, and have led to investigations of the antioxidant properties of nutrients and their influence on bone metabolism.

Polyphenols and anthocyanins are the most abundant antioxidants in the diet and are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate, tea, coffee and wine. Experimental studies in animals or cultured cell lines have supported roles for polyphenols in the preven-

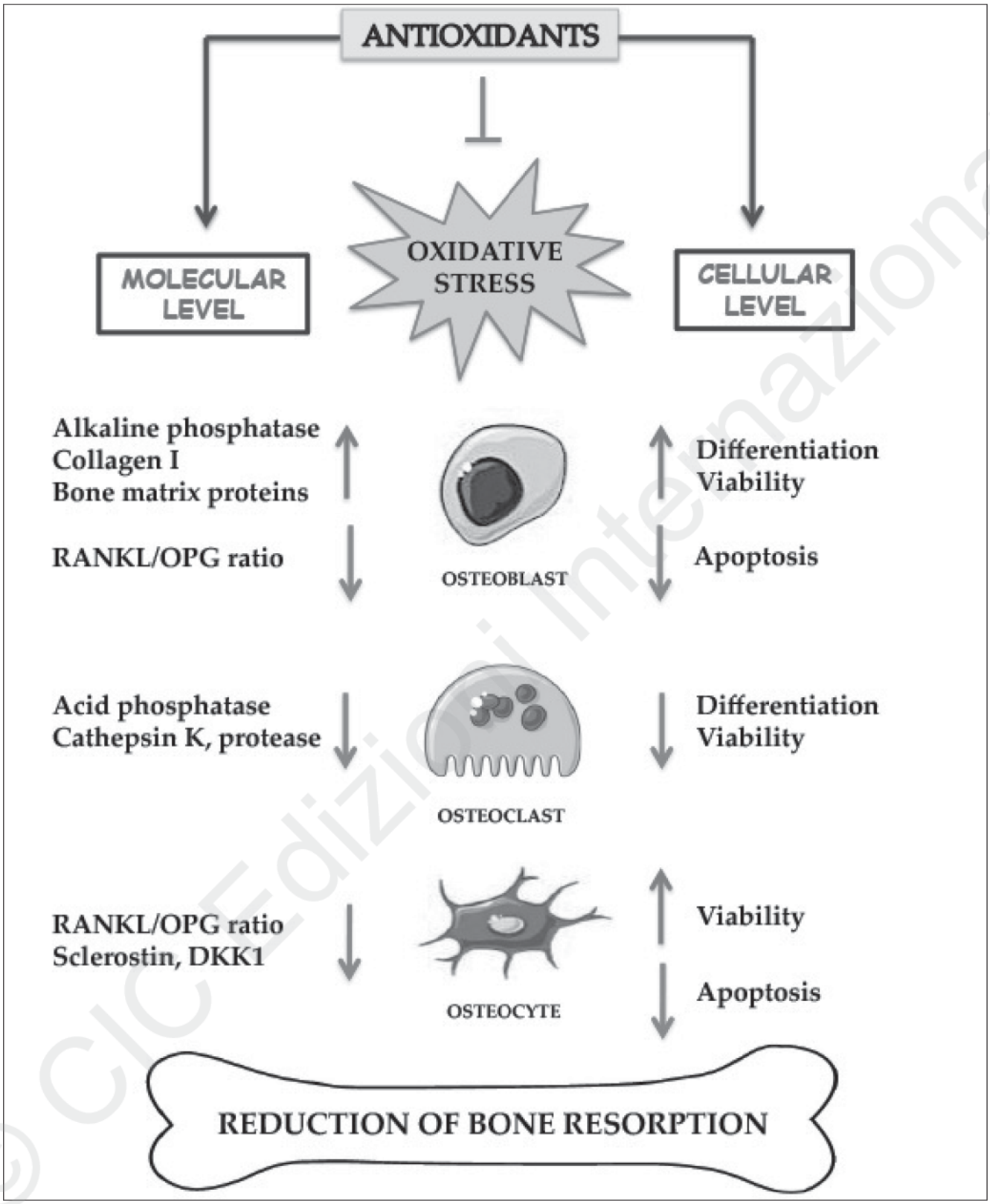

Figure 2 - Effect of antioxidants on bone cells at molecular and cellular level in bone resorption.
Antioxidants inhibit and prevent oxidative stress and affect various enzymes, proteins and cytokines involved in bone remodeling.

tion of cardiovascular diseases, cancer, neurodegenerative diseases, diabetes or osteoporosis (70). Recent data demonstrate that nutritional approaches to antioxidant strategies in bone cells and/or in animals or selected groups of pa-

tients with osteoporosis or inflammatory bone diseases, could be useful for the treatment and prevention of bone loss. In particular, as regards resveratrol, this increases bone mineral density and bone ALP in osteoporotic obese man (71), and represents an effective therapeutic agent in eliminating oxidative stress and in preventing bone loss in ovariectomized and old rats (72). Similarly, dietary supplementation with green tea or Hypericum perforatum or blueberry, containing various types of polyphenols, attenuates trabecular bone loss, prevents loss of collagen in bone matrix, inhibits senescence pathways in osteoblastic cells and prevents osteoporosis in ovariectomized rats (73-76). Tea drinking is also associated with beneficial effects in maintaining bone density in old women and in menopausal women (77, 78). Other data show that green tea polyphenols mitigate bone loss of female rats with chronic inflammation (79), and blueberry extracts have protective effects in acute inflammation and collagen-induced arthritis in the rats (80). Interesting is the protective effect of dietary supplementation of Hypericum perforatum against the oxidative stress and the bone mass loss obtained in rats subjected to forced swimming, in this case it has been shown how antioxidants can induce beneficial effects when oxidative stress and active bone resorption is caused by excessive physical activity (81). Finally, it has been demonstrated in young rats that blueberry phenolic component promotes bone growth activating canonical Want signalling, and diets enriched with blueberries increase bone density mass through suppression of RANKL in stromal cells (82, 83). Indeed, it is note that accumulation of bone mineral during childhood and adolescence is a determining factor for the risk of osteoporosis in aging and in post-menopausal period (84, 85), and various data indicate that daily consumption of fruits or vegetables may be important in increasing the bone mass peak (86, 87).

Conclusions

In this review, it has been shown that changes in intracellular redox state occur during the physiological bone remodeling and that oxidative stress induces important alterations of the differentiation process and activity of bone cells including also the osteocytes. It is highlighted the regulatory role of osteocytes in bone remodeling process and that oxidative stress, related to many diseases including osteoporosis, promotes osteoclast resorption and bone loss through an excessive apoptosis of osteocytes. This is due to RANK signalling activation by increasing RANKL expression with the consequent increase of RANKL/OPG ratio and inhibition of osteoblastic activation and mineralization process. Many *in vitro* and *in vivo* experiments demonstrate that these events are regulated by redox-sensitive signalling pathways in which are involved MAPKs, β-catenin and NF-kB activity. On the contrary, antioxidants have an important role in maintaining a normal bone remodeling process and protecting bone health; in fact, they prevent and/or reduce inflammatory state and bone loss by inhibiting osteocyte apoptosis and mitigating osteoclast activity, consequently, they increase osteoblast activity and induce osteogenesis. Clinical studies show a positive correlation between low levels of antioxidants and bone loss, and this is also related to an increase of markers of bone resorption. Among the biological antioxidants the most important is GSH and different antioxidants act as direct ROS scavengers but also maintaining high levels of intracellular GSH. Moreover, various studies demonstrate also the important role of nutrient antioxidants in bone health, both in young people, in order to favour the formation of optimal peak bone mass, and in the elderly and in menopausal women in order to prevent bone loss, often associated to bone fracture, morbidity and mortality. Indeed, it has been proposed antioxidant use in anti-resorption therapies considering also that they are able to reduce the activity of osteoclasts without determining their destruction which can be important when it is need not only to reduce bone resorption but also to restore physiological bone remodeling. In fact, the anti-resorptive drugs, which are very powerful drugs, currently in use bisphosphonates and the antibody to RANKL, denosumab, block resorption factors, but at the same time reduce the vitality of osteoclasts and promote their apoptosis, breaking the two-way communication between these and osteoblasts, and enabling the restoration of a normal remodeling process (88). Thereafter, it could be interesting to design new therapeutic approaches which include antioxidant treatments for bone diseases related to oxidative stress and bone loss. Indeed, these could act on the redox balance in bone cells and on redox regulated factors and processes involved in bone turnover. However, further studies are needed to clarify the cellular and molecular mechanisms underlying the relationship among oxidative stress, antioxidants and bone metabolism.

Disclosure

All Authors declare that they have no conflicts of interest.

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MMPs, ADAMs and Their Natural Inhibitors in Inflammatory Bowel Disease: Involvement of Oxidative Stress

Filippo Fontani[1], Vladana Domazetovic[1], Tommaso Marcucci[2], Maria Teresa Vincenzini[1] and Teresa Iantomasi[1]*

[1]Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, Italy
[2]Santa Maria Annunziata Hospital, Section of General Surgery, Ponte a Niccheri (Florence), Italy

***Corresponding author:** Teresa Iantomasi, Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, Viale Morgagni, 50, 50134 Firenze, Italy, Tel: 00390552751200, E-mail: tiantomasi@unifi.it*

Abstract

Matrix metalloproteinases (MMPs) are enzymes involved in the degradation of extracellular matrix proteins and in several functions such as wound repair. The regulation of MMPs occurs at various levels including expression and inhibition by tissue specific inhibitors of MMPs (TIMPs). An alteration of intracellular redox state can modulate the activation and/or expression of MMPs. The increased expression and activity of MMPs are related to inflammatory state and pathogenesis of inflammatory bowel disease (IBD). In particular, an imbalance between MMPs and TIMPs is present at intestinal level in IBD, and this can be responsible of alterations of different processes such as repair of damaged mucosa, function and migration of immune cells and mucosal ulceration. A disintegrin and metalloproteinase 17 (ADAM17), belonging to ADAM family, plays an important role in the intestinal inflammation and increases in human colonic mucosa of IBD patients. In this review we report the effects of MMP, ADAM17 and TIMP altered levels on damage amplification caused by inflammation and oxidative stress in IBD. Moreover, the review evidences the role of the intracellular redox state and antioxidants on regulation of expression and activity of some MMPs in condition of oxidative stress in IBD.

Keywords

MMPs, ADAM17, IBD, Oxidative stress, Antioxidants

Introduction

The inflammatory bowel disease (IBD) includes two major forms of chronic intestinal disorders, Crohn's disease (CD) and ulcerative colitis (UC). The exact aetiology of IBD is not well known. There are several factors involved in development of this group of diseases, which include changes in the immune system, bacterial infection and genetic variations [1]. CD is characterized by discontinuous transmural inflammation that, although can occur in any region of gastrointestinal tract, strikes mostly the ileum and the colon. On the contrary, in UC the inflammatory state, typically limited to the mucosa, involves the rectum and may affect a part or the entire colon in a continuous pattern [2,3]. Table 1 shows the highest incidence and prevalence of IBD in principal areas of the world. It is evident that these parameters are more prevalent in highly industrialized nations than in less developed countries [4,5]. The lifestyle and dietary factors affect the development of IBD. In fact, the cigarette smoke enhances the risk for CD and influences negatively the life quality of patients [6,7]. The excessive consumption of milk, animal proteins, carbohydrates and polyunsaturated fatty acids (PUFAs) can enhance the risk for IBD. In particular, starch, generally present in human diet, can promote development of microbic species involved in the induction of autoimmunological reaction in IBD [8]. It has been observed that the imbalance in the consumption of Omega 6 PUFAs and Omega 3 PUFAs is very important in the development of IBD. In fact, Omega 3 PUFAs, differently from Omega 6 PUFAs, do not alter parameters, such as intestinal microbic flora and intestinal permeability, which contribute to IBD pathogenesis [9]. In the development of IBD, an important role is attributed to oxidative stress, due to altered balance between reactive oxygen species (ROS) and antioxidant activity [10]. In particular, the increased oxidative state in intestinal mucosa of CD patients is involved in the chronicization of this pathology [11,12]. Recently, it has been demonstrated that the compounds with antioxidant capacity, associated or not to the traditional therapy for IBD, such as sulfasalazine, azatriopine, corticosteroids, 5-aminosalicylic acid and infliximab, can be beneficial for IBD treatment [13,14]. The

Table 1: Highest annual percent of incidence and prevalence of ulcerative colitis (UC) and Crohn's disease (CD) in the principal areas of the world.

Continent	Incidence	Prevalence
Europe[1]	0.243 (UC) 0.127 (CD)	5.05 (UC) 3.22 (CD)
North America[1]	0.192 (UC) 0.202 (CD)	2.486 (UC) 3.18 (CD)
Asia and Middle East[1]	0.063 (UC) 0.05 (CD)	1.683 (UC) 0.679 (CD)
Australia	0.174 (UC)[1] 0.293 (CD)[1]	0.16 (UC)[2] 0.14 (CD)[2]

[1]Ref. 5; [2]Data from the final IBD report of Australian crohn's and colitis association (ACCA) 2007.

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compounds used in the antioxidant therapy are polyphenols, such as curcumin, resveratrol, quercetin, cathechin, present in plants and in food originating from plants [15]. Also food rich in micronutrients with antioxidant properties, such a vitamin E and C, can ameliorate ROS-induced symptoms [16]. Proteinases, such as matrix metalloproteinases (MMPs), and A disintegrin and metalloproteinase 17 (ADAM17), belonging to ADAM metalloproteinases, affect the intestinal tissue damages and the inflammatory processes which characterize IBD [17-19]. In fact, the oxidative stress can play a crucial role in the modulation of MMPs activity and expression [20]. MMPs are involved principally in the turnover of extracellular matrix, while ADAM17, a membrane-bound enzyme, cleaves cell surface proteins such as cytokines and cytokine receptors [21]. Deregulated activity and expression of MMPs affect cell adhesion, immune cell migration, cytokine production and impairs wound healing [22]. The activities of MMPs are closely regulated at various levels such as transcription, activation of zymogens and inhibition by endogenous inhibitors named tissue inhibitors of MMPs (TIMPs) [23,24]. TIMPs reduce MMPs activity, by forming non covalent 1:1 stoichiometric complex, [25] but they also affect, through their specific receptor, cell growth, apoptosis, differentiation and angiogenesis [26]. The balance between MMPs and TIMPs is very important in the inflammatory response and in the outcome of tissue injury. In fact, an increased MMPs/TIMPs ratio has been found in inflamed colon of IBD [27], and an enhanced activity of MMPs may amplify intestinal immune response by recruiting the inflammatory cells [28]. Given that, it has been suggested the potential use of inhibitors of MMPs in IBD treatment as demonstrated in IBD animal models in which the administration of several MMP inhibitors reduces the inflammatory state [29,30]. Moreover, some data indicate that the antioxidant compounds may have a therapeutic effect by reducing the altered levels of MMPs detected in intestinal subepithelial myofibroblasts (ISEMFs) isolated from colonic mucosa of CD patients (CD-ISEMFs) [31,32]. An increase of ADAM17 has been also found in human colonic mucosa of IBD patients [33] and this suggests that ADAM17 inhibition may be an alternative strategy to block signalling pathway of tumor necrosis factor (TNF-α), a cytokine involved in intestinal inflammation.

Metalloproteinases

MMPs, collectively called matrixins, participate in the modulation of homeostasis of extracellular matrix (ECM) by cleaving ECM proteins such as collagen, fibronectin, elastin and laminin [34]. However, even if MMPs are involved in the normal tissue remodelling, they also act on substrates different from ECM proteins, regulating many physiological functions such as cell proliferation, adhesion, migration, growth factor bioavailability, chemotaxis and signalling [34]. Under normal physiological conditions, MMPs are present in the latent form and at low concentration. They generally present a prodomain, a catalytic domain, a hinge region and a hemopexin domain [34]. To date 23 MMPs have been found and identified in humans. On the basis of substrate specificity, sequence similarity and domain organization, MMPs can be divided into: Collagenases, Gelatinases, Stromelysins, Matrilysins, Membrane-Type MMPs. These last MMPs distinguish in transmembrane or glycosylphosphatidylisositol- (GPI)-anchored MMPs (Table 2). Seven MMPs are not classified in the above categories and have been indicated as other MMPs. In particular, MMP-12 (metalloelastase), mainly expressed in macrophages, is essential for their migration and digests, besides elastin, a number of other proteins [35,36]. MMP-19 is identified as T-cell-derived autoantigen from patients with rheumatoid arthritis [37]. MMP-20, located within newly formed tooth enamel, is also called enamelysin and digests primarily amelogenin [38]. MMP-21 has been detected in neuronal tissues [39], in cancer and fetal tissue [40], indicating a role in human embryogenesis and tumor progression. MMP-22 function is still not known, but its substrates "*in vitro*" are gelatin and casein [41]. The characterization of MMP-21 and MPP-22 genes showed almost identical sequences [42]. MMP-23, mainly expressed in reproductive tissues, is a membrane-anchored protein with four domains. Differently to other MMPs, it lacks the cysteine switch motif in the prodomain and also the hemopexin domain. Instead, it has a cysteine-rich domain followed by an immunoglobulin-like domain [43]. The latest member added to MMP family is MMP-28, epilysin, initially found in keratinocytes. It is expressed in a number

Table 2: Classification of different MMPs on substrate specificity.

MMPs			Substrates
Collagenases		***MMP-1 (Collagenase 1)***	Collagens type I, II, III,VII, VIII, X and XI, gelatin, fribronectin, vitronectin, laminin, entactin, tenascin, aggrecan, link protein, myelin basic protein, versican
		MMP-8 (Collagenase 2)	Collagens type I, II and III, aggrecan
		MMP-13 (Collagenase 3)	Collagens type I, II, III, IV, VI, IX, X and XIV, collagen telopeptides, gelatin, fibronectin, SPARC, aggrecan, perlecan, large tenascin-C
		MMP-18 (Collagenase 4)	Collagen type I (rat)
Gelatinases		***MMP-2 (Gelatinase A)***	Collagens type I, II, III, IV, V, VII, X and XI, gelatin, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC, aggrecan, link protein, galectin-3, versican, decorin, myelin basic protein
		MMP-9 (Gelatinase B)	Collagens type IV, V, X and XIV, gelatin, elastin, vitronectin, laminin, entactin, tenascin, SPARC, aggrecan, link protein, galectin-3, versican, decorin, myelin basic protein
Stromelysins		***MMP-3 (Stromelysin 1)***	Collagens type III, IV, IX, X and XI, collagen telopeptides, gelatin, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC, aggrecan, link protein, decorin, myelin basic protein, perlecan, versican, fibulin
		MMP-10 (Stromelysin 2)	Collagens type III, IV and V, gelatin, elastin, fibronectin, aggrecan, link protein
		MMP-11 (Stromelysin 3)	Collagens type IV, gelatin, fibronectin, laminin
Matrilysins		***MMP-7 (Matrilysin 1)***	Collagens type I and IV, gelatin, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC, aggrecan, link protein, decorin, myelin basic protein, fibulin, versican
		MMP-26 (Matrilysin 2)	Collagens type IV, gelatin, fibronectin, vitronectin
Membrane-type MMPs	**Trans-membrane**	***MMP-14 (MT1-MMP)***	Collagens type I, II and III, gelatin, fibronectin, tenascin, vitronectin, laminin, entactin, aggrecan, perlecan
		MMP-15 (MT2-MMP)	Fibronectin, tenascin, entactin, laminin
		MMP-16 (MT3-MMP)	Collagens type III, gelatin, fibronectin, vitronectin, laminin
		MMP-24 (MT4-MMP)	Fibronectin, gelatin, chondroitin sulphate proteoglycan, dermatan sulphate proteoglycan
	GPI-anchored	***MMP-14 (MT1-MMP)***	Gelatin
		MMP-15 (MT2-MMP)	Collagens type IV fibronectin, gelatin, chondroitin sulphate proteoglycan, dermatan sulphate proteoglycan

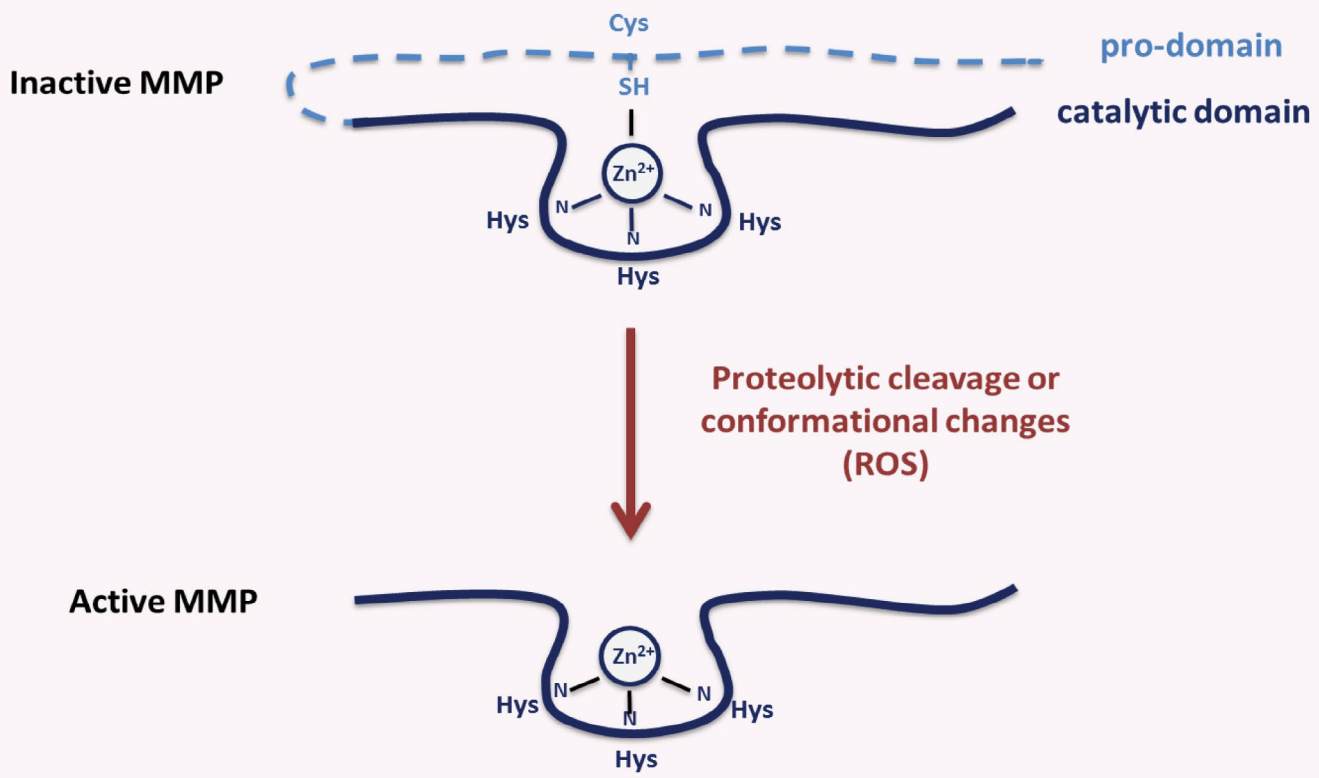

Figure 1: MMP activation: involvement of "cysteine-switch" mechanism.

MMPs are activated through pro-domain proteolytic cleavage or conformational changes. Reactive oxygen species (ROS) break thiol-Zn2+ interaction and induce conformational changes which favor MMP activation.

of normal tissues, suggesting that it regulates tissue homeostasis [44]. Enhanced expression of epilysin has been observed in basal keratinocytes during wound healing; in fact, expression patterns in intact and damaged skin suggest that MMP-28 might function both in tissue homeostasis and wound repair [45].

Regulation of MMPs

Catalytic activity of MMPs is regulated at four levels: gene expression, compartmentalization, proenzyme activation, enzyme inactivation. Moreover, it is further controlled by substrate availability and affinity. Some MMP members, such as MMP-2, MMP-19, MMP-28 and several MT-MMPs, are expressed in normal tissue, being involved in tissue homeostasis. However, most MMPs are induced in tissue repair or remodelling subsequently to diseases or inflammatory processes. For the most part, the production of MMPs is regulated at transcription level by specific signals that are temporally limited and spatially confined [34]. MMPs are secreted as proenzymes and are activated in the pericellular and extracellular space. One-third of MMPs contains or RRKR sequence between the pro and catalytic domain, which serves as a target sequence for proprotein convertases (PPCs), a family of proteins that activate other proteins. One of the most well-known PPCs is Furin, a subtilisin-like serine protease present in trans-Golgi network [46], which cleaves protein precursors on motifs such as Arg-X-X-Arg and Lys/Arg-Arg. In fact, MMPs with a furin cleavage site are processed intracellularly before secretion [47,48].

In MMP prodomain is present a conserved cysteine that interacts by its thiol group with the zinc ion of the catalytic site. In this way, ProMMPs are kept in the inactive state and become catalytically active after disruption of the thiol-Zn2+ interaction [34]. This process, named the "cysteine-switch", represents a general and required step in the activation of all proMMPs [49]. The break of thiol-Zn2+ interaction can be due to the proteolytic activity of other proteinases that directly remove the prodomain. ROS, SH-reactive agents, or detergents can also induce conformational changes of the proenzyme, thus favouring conversion to an activated state by autolytic cleavage of the prodomain [50] (Figure 1). However, allosteric perturbation, due to binding of proMMPs to macromolecules, such as integrins, gelatin, collagen, can activate MMPs in their intact or partially cleaved form [51]. In fact, in the cysteine-switch mechanism, the removal of prodomain is not necessary for the activation of proMMPs; differently, the elimination of zinc-thiol interaction is required. Oxidants firstly activate, by oxidating the thiol prodomain, and subsequently inactivate MMPs, by modifying amino acids crucial for their catalytic activity. In particular, ROS, by modifying cysteine SH-group, activate proMMPs via autolytic cleavage "*in vitro*" [52]. However, this ROS effect occurs at low concentrations; in fact, high concentrations of ROS induce the inactivation of MMPs [53]. Also S-glutathionylation of the cysteine in the conserved domain of propeptide that occurs in the presence of peroxynitrite and glutathione (GSH), activates MMPs without cleavage of prodomain [54]. On the contrary, other studies show that MMPs activation, due to peroxynitrite alone or with GSH, is followed by the cutting of prodomain [55], indicating that both mechanisms are possible.

The local tissue activities of MMPs are also modulated by TIMPs, specific inhibitors of matrixins [56,57]. Four TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified in vertebrates [58], and their expression is regulated during development and tissue remodelling. Under pathological conditions, changes in TIMP levels are very important considering that they directly affect and unbalance MMP activity. TIMPs have an N- and C- terminal domain of about 125 and 65 amino acids respectively, each containing three conserved disulfide bonds [59,60]. The N-terminal domain folds as a separate unit and is able to inhibit MMPs [59]. TIMP-1 structure and its inhibition mechanism were determined by X-ray crystallographic studies of the TIMP-1-MMP-3 complex [61]. TIMPs inhibit all MMPs tested so far, except for TIMP-1 that failed to inhibit MT1-MMP [62].

MMPs in IBD

Collagenases (MMP-1, MMP-13)

An increased expression of MMP-1, related to degree of inflammation, has been detected in biopsies from colonic mucosa of

patients with active IBD [27,63]. In particular, MMP-1 expression increases by 230-fold in ulcerated colonic mucosa of IBD patients as compared to normal controls. Moreover, the increase of MMP-1 is attributed principally to macrophages present within the inflamed mucosa [27]. The enhanced expression of MMP-1 potentiates the inflammatory response in IBD intestinal ulcers [64] and in CD granulation tissue [65,66], suggesting that MMP-1 plays an important role in the increase of inflammatory state. The increased expression of MMP-1 in the colonic mucosa of UC patients, related to the enhancement of MMP-1 plasma levels [67], induced acute tissue injury and initial steps of ulceration due to an excessive cleavage of ECM proteins [68]. Indeed, the expression of MMP-1 is greatly increased in ulcerated and inflamed colon areas and in serum of UC patients [67,69-71]. All together, these results suggest that MMP-1 expression and release correlate with UC severity and mucosal damage. Analysis of the relationship between MMP-1 expression and the severity of the disease shows that the expression of MMP-1 in patients with moderate UC is significantly higher than that in patients with mild UC, demonstrating that MMP-1 can be used as biomarker to judge the severity of clinical symptoms in patients. An enhanced expression of MMP-1 up-regulates TIMP-1 in ulcerated mucosa of UC patients, but this leads to an imbalance between MMP-1 mRNA and TIMP1 mRNA levels. In reality, TIMP-1 mRNA levels do not counteract those of MMP-1 mRNA with consequent overdegradation of ECM in UC [68].

MMP-13 is another collagenase that has as substrate also pro-MMP-9, and is considered important in IBD being strongly expressed in fibrotic areas of chronic cutaneous ulcers in IBD patients. In particular, it is detected only in fibroblasts of the ulcer bed, for this it could be involved in remodelling of the submucosal matrix [72]. The pro-inflammatory role of MMP-13 is due to its ability to release TNF-α from membranes [73]. In fact, it has been demonstrated that both the administration of dextran sulphate sodium (DSS) in rats, in order to mimic colitis clinical conditions, and lipopolysaccharide (LPS)-induced sepsis up-regulate MMP-13 levels involved in the TNF-α cleavage [74]. The formation of bioactive TNF-α causes alterations of intestinal epithelial barrier integrity by increasing intestinal permeability and destabilizing tight junctions [74]. To our knowledge there are no data about the role of oxidative stress on collagenase activity in IBD. However, some data in literature show that an increase of MMP-1 expression and activity, related to ROS production, occur in a variety of cell lines and tissues [75]. Also buthionine sulfoximine (BSO), inhibitor of GSH synthesis, and aminotriazol, catalase inhibitor, increase MMP-1 expression [76]. MMP-13 is overexpressed in osteoarthritic chondrocytes when an increase of ROS production occurs [77]. On the contrary, antioxidants such as the polyphenol epigallocatechin-3-gallate, present in green tea, GSH, Vitamin A and E decrease MMP-1 expression in liver fibrosis, in transformed human heart fibroblasts and in a porcine model of atherosclerosis [78-80]. Moreover, resveratrol, polyphenolic compound present in red wine with anti-inflammatory and antioxidant properties [81], inhibits production of MMP-1 and stromelysin MMP-3 in human chondrocytes [82], and MMP-13 expression in osteoarthritic rats [83].

Gelatinases (MMP-2, MMP-9)

MMP-2 is expressed in normal colon, mainly in the epithelial cells and in lamina propria. An increase of MMP-2 activity was detected in IBD [17,63] and in particular in CD intestinal fistulae [84]. In fact, the increased proteolytic activity of MMP-2 can have an important role in fistulae formation by degrading the basement membrane that supports endothelial and epithelial cells in the gut [84]. The epithelial barrier dysfunction characterizes the pathogenesis of intestinal inflammation and MMP-2 represents a key factor for intestinal barrier functionality [85,86]. However, the role of MMP-2 in the development of colitis is still unclear. Some authors demonstrated that MMP-2 plays an important protective role in maintenance of the integrity of intestinal epithelial barrier [87]. They show that in experimental animal models of colitis, induced by chemicals or bacteria, MMP-2-/- mice develop the acute colitis much more intensely than WT mice [87]. Myofibroblasts are an important source of MMP-2 in intestine during the inflammatory state [84]. An increase of MMP-2 secretion and activation, related to decrease glutathione/oxidized glutathione ratio (GSH/GSSG) and inflammatory state, is detected in CD-ISEMFs [31]. In these cells, N-acetylcysteine (NAC), a precursor of GSH synthesis, down-regulates MMP-2 secretion through the increase of GSH/GSSG ratio and exhibits a direct inhibitory action on MMP-2 activity. Moreover, NAC is also able to reduce MMP-2 activation induced by TNF-α thus restoring the physiological activity of enzyme in CD-ISEMFs [31]. Redox regulation of MMP-2 secretion involves c-jun N-terminal kinase (JNK) pathway [31] that phosphorylates transcriptional factors present in most MMP promoters [88]. All data on MMP-2 indicate the importance of correct regulation of production and activity of this MMP. In fact, the lack of MMP-2 can be responsible for deregulation of intestinal barrier function; on the contrary, the excess of MMP-2 can induce fistulae formation. The role of oxidative stress on secretion and activation of MMP-2 has been confirmed in CCD-18Co cells (18Co), a myofibroblast cell line derived from human colonic mucosa, in which the intracellular redox state was modulated through BSO or NAC [31].

High levels of MMP-9 are also present in several animal models of colitis and in human IBD [63,89]. Experimental DSS-induced colitis in rats increases pro-MMP-9 activity [29]. The synthetic inhibitor of MMPs, CGS-27023, administrated to these rats for 14 days, increases mucosal repair process and reduces inflammation, indicating a close relationship between pro-MMP-9 activity and injury in inflamed colonic tissue [29]. MMP-9 is the most abundantly expressed protease that correlates with disease activity in IBD [17,90], and it is associated with fistulae in acute CD [17,91]. It has been demonstrated, by immunohistochemical analysis, that MMP-9 is expressed abundantly in fistulae of patients with CD, but only in those with prominent acute inflammation [84]. In the fistulae formation, MMP-9 could have a similar role to that of MMP-2; however, MMP-9 may promote tissue injury by inducing extravasation of neutrophils in areas with acute inflammation and by accelerating the proteolysis of matrix proteins partly degraded by other MMPs [91]. These tissue damages play a pivotal role in IBD; in fact, differently to that occurs for MMP-2, MMP-9-/- mice do not develop colitis due to bacteria or chemicals [86] and are resistant to inflammatory processes [29,92,93]. MMP-9 is involved in the inflammatory response by delaying re-epithelialization processes [94], impairing wound healing [95], increasing endothelial permeability [96,97], and activating several proteins including fibrinogen, interleukin (IL)-1β, IL-8, and transforming growth factor-β (TGF-β) [95,98-100]. It is shown that serum concentration of MMP-9 is higher in UC and CD patients than that measured in control serum, and MMP-9 levels correlate well with the disease activity [101]. These data were confirmed by the demonstration that a significant increase of MMP-9 occurs in active CD and UC as compared with MMP-9 levels detected in controls and in patients with inactive forms [102]. The correlation between MMP-9 serum levels and the clinical activity of disease was observed also in children affected by IBD [103,104]. Fecal MMP-9 levels increase in IBD patients but this increase is higher in UC than in CD [105]. These results suggest that serum and fecal MMP-9 levels can be considered as supportive markers for disease activity or for diagnosis of different types of IBD, respectively. An up-regulation of MMP-9 expression has been associated with the release of vascular endothelial growth factor (VEGF) in animal models of colitis [106]. In fact, a strong correlation between circulating VEGF-A and serum MMP-9 in CD patients was observed [102]. MMP-9 seems to be involved in angiogenesis by recruitment of pericytes to the new vessels [107]. In this process platelet-derived growth factor (PDGF), whose availability as well as biological functions are regulated by its binding to ECM, plays the major role. Given that a correlation between the concentration of this growth factor and MMP-9 levels in both CD and UC was found, it has been speculated that MMP-9 might participate in the angiogenesis through the release of PDGF from ECM or degradation of ECM elements involved in PDGF binding [102]. Recent data demonstrate that aerial part extracts of *Lavandula dentata* and *Lavandula stoechas* improve the redox state by increasing GSH levels

in 2,4,6-trinitrobenzene-sulfonic-acid (TNBS)-induced rat colitis model [108]. Moreover, this increase of redox state is associated to down-regulation of MMP-9 expression [108].

Stromelysins (MMP-3, MMP-10)

MMP-3 is induced consistently in several animal models [109] and in human IBD [63]. It is expressed during IBD in mononuclear macrophage-like cells and in lamina propria stromal cells below the ulcerated areas [38,72,110]. An up-regulation of MMP-3 is present in CD [65,110] and, similarly to gelatinase MMP-9, it is related to mucosal damage and fistulae formation through degradation of the matrix in areas with active inflammation [17,73,91]. MMP-3 and MMP-9 concentrations in sera of pediatric patients with IBD reflect various stages of disease activity. In fact, significantly higher concentrations of these MMPs are present in the sera of patients with the active form of disease as compared to those found in sera of patients with the mild form or in control group [103,104]. Therefore, the levels of MMP-3 and MMP-9 might be useful in the clinical evaluation of patients. A marked overexpression of MMP-3 was assessed by mRNA levels in inflamed mucosal samples from patients with UC as compared to controls [27]. These results are also supported by data that demonstrate an overexpression of MMP-3 in intestinal inflamed sites of children with IBD [111]. Functional experiments in an *ex vivo* human foetal small intestine explant model have clearly shown that MMP-3 also acts as a key mediator of T cells, and TNF-α mediates tissue injury in the gut [112-114]. The increase of oxidative state present in CD-ISEMFs is responsible for the increased MMP-3 production detected in these cells stimulated or not with TNF-α, as compared to the control ISEMFs [32]. In fact, NAC restores MMP-3 levels in CD-ISEMFs to those of control ISEMFs. Data obtained in 18Co treated with BSO and/or NAC or curcumin, antioxidant present in *Curcuma Longa* with anti-inflammatory effect [115], highlight the regulatory role of antioxidants on production of MMP-3. However, their effect is not closely related to changes of oxidative state, suggesting that antioxidants affect MMP-3 production also by their direct action on transcriptional factors [32]. Indeed, mitogen activated protein kinases (MAPKs), activated in the presence of oxidative stress, are involved in the modulation of MMP-3 levels [31,32]. Curcumin decreases MMP-3 expression both by increasing GSH levels and by inhibition of p38 mitogen activated protein kinase (p38 MAPK) signalling pathway and transcriptional factors, such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) [116]. Moreover, curcumin reduces MMP-3 in biopsies and ISEMFs of adult and children affected by IBD [117] indicating the role of oxidative stress in the increased activity of MMPs in these patients.

MMP-10 together with matrilysin MMP-7 plays a role in intestinal wound healing [118], and MMP-10 is actively expressed in epithelial cells at the ulcer margin. This location and the finding that the same cells produce laminin-5 suggest an important role for MMP-10 in epithelial cell migration [72]. In addition, MMP-10 is also expressed in macrophage and lymphocyte-like cells in areas of inflammation but not in T lymphocytes. In fact, T lymphocytes do not express MMP-10 in the intestine but stimulate other cells of lamina propria to express it, participating in formation of early lesions in IBD [72,112].

Matrilysin (MMP-7, MMP-26)

MMP-7, expressed in many adult tissues, is implicated in innate intestinal defence [119]. Cytokines and growth factors, such as IL-1, epidermal growth factor (EGF) and TNF-α, at elevated concentrations at inflammatory sites, may induce the synthesis of MMP-7 [120]. The expression of this MMP is an important hallmark of surface epithelium at the edge of gastrointestinal ulcers. In fact, MMP-7 was not detected in intact tissue distant from the wound [65] and in normal gastric or colonic mucosa [121,122]. MMP-7 mRNA was detected in crypt abscesses associated with idiopathic IBD [65]. Its expression also increases in IBD and its levels correlate with disease activity in UC [123,124]. During IBD, MMP-7 has been detected in areas surrounding ulcers, suggesting a role in repair/wound healing. Recently, it has been demonstrated that the glandular epithelium MMP-7 correlates with the disease incidence in UC and with the lesion location in CD [125].

MMP-26, specifically expressed in epithelial carcinomas and

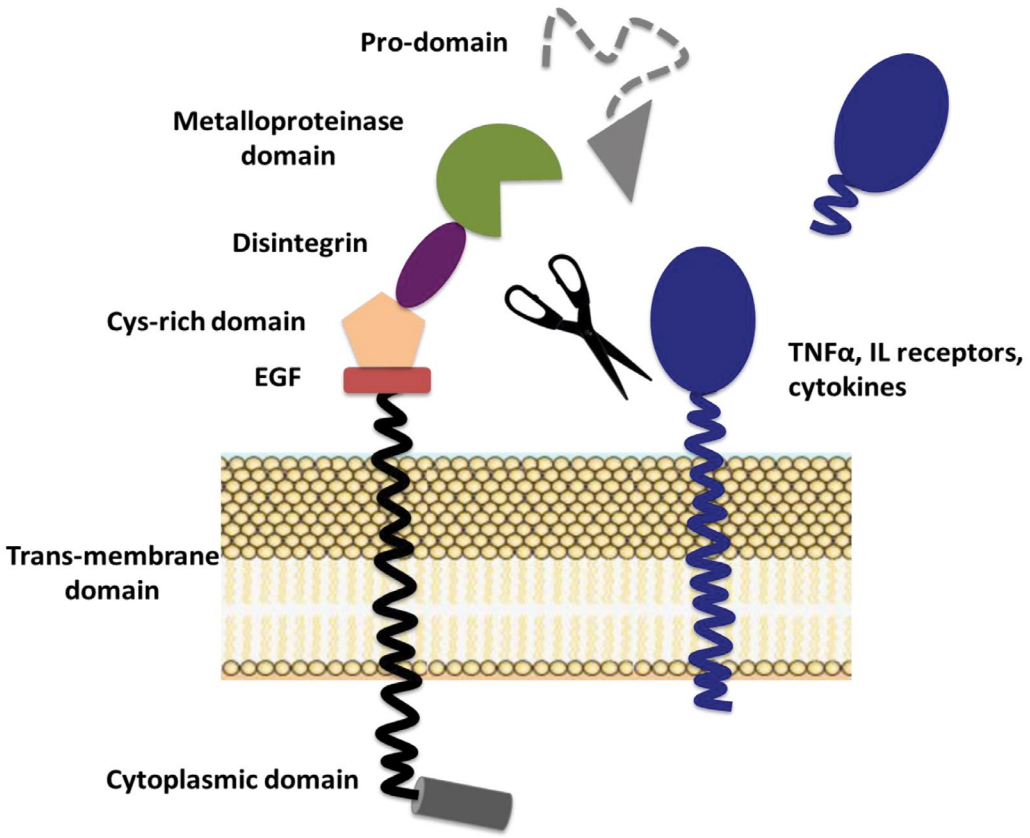

Figure 2: Schematic structure and activation of ADAM members.
Members of ADAM family after activation cleave the substrates from the cell surface.

in normal adult kidney, decreases after anti-TNF-α treatments in intestinal neutrophils of CD patients [126]. This suggests the involvement of MMP-26 in tissue destruction or migration of neutrophils. MMP-26 is also expressed in migrating enterocytes localized next to the intestinal ulcer in IBD [127].

Other MMPs (MMP-12, MMP-19, MMP-28)

MMP-12 increases in the immune response in lamina propria macrophages [72,128], and this proteinase may have a role in macrophage migration and tissue inflammation in CD [128]. MMP-19, expressed in non-migrating enterocytes in IBD intestine [127], seems to be important for appropriate immune response in colitis. In fact, in DSS-induced colitis the lack of MMP-19 causes an increase of inflammatory state and mucosal damage in mice [129]. MMP-28, differently to previously described MMPs, decreases in inflamed mucosa in UC [130], suggesting that this MMP is not involved in IBD intestinal damage [127].

ADAM17 and its regulation

The differences between MMPs and ADAMs consist in domain structure and in particular in the sequence of catalytic domain. The ADAM structure contains a prodomain, a metalloproteinase catalytic domain, a disintegrin domain, an EGF-like (cysteine-rich) domain, a single transmembrane domain and a cytoplasmic portion [21] (Figure 2). Members of ADAM family have emerged as major ectodomain shedding proteinases, which release a large number of molecules in various physiological conditions. However, the uncontrolled release of some substrates is related to various pathological processes. ADAM17, also known as tumor necrosis factor-α converting enzyme (TACE), is a membrane-bound enzyme belonging to ADAM family that cleaves cell surface proteins such as cytokines (TNF-α) or cytokine receptors (IL-6-receptor and TNF-receptor). Considering the effect of ADAM17 on TNF-α or IL-6, it has been speculated that its inhibition could improve autoimmune diseases [131]. In fact, membrane-bound TNF-α acquires the pro-inflammatory activity after its cleavage by ADAM17 [132]. Moreover, the shedding of IL-6R from membrane induces IL-6 trans-signalling on cells expressing only IL-6 receptor chain gp130, thus increasing the inflammatory state with consequent cancer risk [133,134]. Phorbol ester, phorbol 12-myristate 13-acetate (PMA), is a potent activator of ADAM17 by protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) activation. In fact, the inhibition of these kinases decreases ADAM17 activity [135]. ROS induce an increase of ADAM17 activity through direct modification of disulphide bonds that allows ADAM17 to assume an open active conformation [136] and also mediate up-regulation of ADAM17 expression [137,138]. The compartmentalization plays an important role in the regulation of ADAM17. In fact, it is stored in perinuclear region and its translocation on cell membrane occurs after phosphorylation of Thr 735 modulated by p38 MAPK activation [139]. Apoptosis also induces, probably by activation of p38 MAPK, an increase of ADAM17 activity and consequent translocation on cell surface [140]. ADAM17 activity is down-regulated by the natural tissue inhibitor TIMP-3 [141], and it has been demonstrated that also Tetraspanin CD9 negatively regulates ADAM17-mediated TNF-α shedding as well as intercellular adhesion molecule-1 on the cell surface [142].

Members of adam family in IBD (ADAM9, ADAM10, ADAM17, ADAM19)

ADAM17, normally expressed in the human colonic mucosa, increases in human IBD [33] and its expression is up-regulated by TNF-α in endothelial cells [143]. Transmigration of polymorphomononuclear leukocytes (PMNL) across epithelia is the primary event of acute phase in CD, and it can be responsible for the TNF-α secretion by intestinal cells [144]. Moreover, in this phase of acute inflammation, an increase of the expression of ADAM17, but not of its inhibitor TIMP-3, occurs in intestinal endothelial cells (IEC) [145]. All these data demonstrate that the early upregulation of ADAM17 was linked to TNF-α production during PMNL transepithelial migration. Reduced levels of ADAM17 increase sensitivity to colitis in mice [146] and studies, performed in DSS-induced colitis, demonstrate that epithelial ADAM17 is very important to provide colitis resistance in rats [147]. In fact, it is possible that ADAM17, through the activation of EGFR and its signalling pathways, promotes mucosa repair by inducing epithelial cells proliferation and globet cell differentiation [147]. Some data show that the ADAM17 expression levels, detected in epithelial cells and in intestinal biopsies derived from intestinal mucosa of IBD patients, were not significantly different from ADAM17 values measured in control samples [148,149]. On the contrary, other data demonstrate that ADAM17 expression enhances in CD patients and promotes neutrophil migration and colitis [145]. It is possible that the differences highlighted in these studies may be due to different inflammatory state of intestinal mucosa and/or therapy used. In fact, ADAM17 levels can be related to oxidative stress considering that an increase of this protease occurs in intestinal myofibroblast cell line in which is present an increase of ROS due to BSO and/or TNF-α treatment [138]. The involvement of ADAM17 in IBD is also observed in mice with DSS-induced colitis, in which TACE significantly increases during the period of DSS treatment and returns to normal levels in the remission phase. Moreover, the treatment with a specific inhibitor of ADAM17, compound 11p, reduces clinical signs of DSS-induced colitis maintaining the integrity of colon mucosa and avoiding the infiltration of inflammatory cells [150]. Resveratrol ameliorates experimental colitis by reducing ADAM17 and increasing TIMP-3 expression through the activation of the deacetylase, silent information regulator-1 (SIRT-1) [150]. ADAM17 can be considered a target in therapies for chronic inflammatory diseases and compounds able to inhibit ADAM17 activity have already been developed [151].

Although ADAM17 is the member of ADAM family highly expressed in IBD, it has been demonstrated that ADAM19 is also up-regulated in mucosa of IBD patients [152]. The major increase of ADAM19 is detected in patients with UC and its expression is stimulated by TNF-α and various cytokines. Differently, no variation of ADAM9 and ADAM10 expression between IBD patients and control was observed [152].

TIMPs in IBD (TIMP-1, TIMP-2, TIMP-3, TIMP-4)

TIMP-1: Contradictory data are reported in literature about the production of TIMP-1 in intestinal inflamed mucosa. In IBD it has been demonstrated that TIMP-1, expressed by inflammatory cells, activated fibroblasts and vascular smooth muscle cells, increases predominantly in the areas of mucosa with active inflammation and, in particular, at ulcer bases [64]. This localization suggests that TIMP-1, along with the increase of MMP-1, plays a role in the tissue reorganization after mucosal damage. An increased expression of TIMP-1, related to disease severity, is observed in inflamed intestinal area of UC patients [69], as well as in inflamed and especially ulcerated colon mucosa of IBD patients [27]. In a study using cultures of biopsies isolated from intestinal mucosa of IBD patients, TIMP-1 levels were higher in inflamed mucosa of both CD and UC as compared with uninflamed mucosa [153]. Moreover, TIMP-1 showed a strong correlation with levels of proinflammatory cytokines such as IL-6, IL-1β and IL-10 [153]. An enhancement of TIMP-1 has been observed both in inflamed intestinal mucosa and in serum of UC patients [71]. In particular, it has been observed that TIMP-1 plasma levels in UC patients correlate positively with scored endoscopic degree of mucosal injury, with indices of disease and clinical activity, and with C reactive protein concentration, a marker of inflammation levels. Therefore, TIMP-1 plasma concentration may be a possible biomarker of disease activity [71]. No increase of TIMP-1 production has been detected in CD-ISEMFs type cells stimulated or not with TNF-α as compared to C-ISEMFs [32]. Similar results were obtained in 18Co treated with BSO and/or NAC indicating that the intracellular redox state does not affect the production of this inhibitor [32]. Indeed, an increased secretion of TIMP-1 is measured only in fibrotic myofibroblasts and is associated with the presence of fibrotic structures in CD [154]. Moreover, it has also been observed that TIMP-1 production enhances in myofibroblasts of CD patients only after treatment with infliximab (chimeric monoclonal antihuman TNF-α antibody), and

Table 3: MMPs, ADAMs and TIMPs expression and role in inflammatory bowel disease (IBD).

MMPsG3:I20, ADAMs and TIMPs	Expression in IBD	Role in IBD
MMP-1	↑	Potentiates the in lammatory response Ulceration (UC) Biomarker of disease stage (UC)
MMP-13	↑	TNFα cleavage Mucosal damage
MMP-2	↑	Maintenance of intestinal epithelial barrier integrity Fistulae formation (CD)
MMP-9	↑	Mucosal damage Fistulae formation (CD) Angiogenesis Supportive biomarker for diagnosis of UC and CD and the disease stage
MMP-3	↑	Mucosal damage (UC) Fistulae formation (CD) Biomarker of disease stage
MMP-10	↑	Intestinal wound healing
MMP-7	↑	Intestinal wound healing. Biomarker of disease stage in UC
MMP-26	↑	Mucosal damage and neutrophil migration
MMP-12	↑	Immune response
MMP-19	**N.D.**	Increase of in lammatory state and mucosal damage
MMP-28	↓	Not involved.
ADAM-17	↑	TNFα cleavage Mucosa repair Colitis resistance? Neutrophil migration and colitis?
TIMP-1	↑	Biomarker of disease stage
TIMP-2	**?**	Not involved
TIMP-3	↓	TNFα cleavage deregulation
TIMP-4	↓	Increase of MMP-2 activity Increase of platelet aggregation

↑: Increase; ↓**:** Decrease; **?:** Contradictory data; **N.D.:** No data to our knowledge.

MMP: Matrix Metalloproteinase; ADAM: A Disintegrin and Metalloproteinase; TIMP: Tissue Inhibitors of MMPs; UC: Ulcerative Colitis; CD: Crohn's Disease.

this effect is not accompanied by significant induction of apoptotic process or alterations in MMP expression [155]. Considering these findings, it has been hypothesized that not myofibroblasts but other inflammatory cells, such as lymphocytes and macrophages, may be the responsible source for the increased levels of TIMP-1 in intestinal mucosa of CD patients [27,32].

TIMP-2: No statistical difference in TIMP-2 levels was found between IBD patients and healthy controls in serum [156]. Moreover, no remarkable expression of TIMP-2 is observed in inflamed mucosa of IBD patients [156]. TIMP-2 mRNA levels, measured by PCR in biopsies from IBD patients, remain unchanged in inflamed and uninflamed mucosa [27]. On the contrary, other studies demonstrated an increase of TIMP-2 serum levels in UC and CD patients, suggesting its use as a potential marker for IBD activity [157].

TIMP-3: TIMP-3, present in both uninflamed and inflamed gut mucosa and predominantly expressed in macrophage-like cells or fibroblastic-like cells in lamina propria and in endothelial cells [72], inhibits the inflammatory response as shown by studies based on timp3-/- mice or gene transfer "*in vitro*". TIMP-3 is essential for innate immune function and has a pivotal role in the regulation of inflammatory response [158]. In fact, the loss of this TIMP causes deregulation of cleavage of TNF-α and its receptors by inactivation of ADAM17 in innate immunity [158]. Very severe colitis, associated with increased expression of inflammatory cytokines, has been determined in TIMP-3-deficient mice after treatment with TNBS [149]. In contrast, transgenic mice for TIMP-3 express less cytokines and are largely resistant against TNBS-induced colitis. In addition, it has been demonstrated that TIMP-3 regulates negatively not only ADAM17 but also functional activity of MMPs that are increased in IBD [149]. For this reason, given that MMP-9 derived from epithelium has a crucial role in the induction of intestinal damage, the severe intestinal damage seen in phenotype TIMP-3-KO mice after TNBS administration, may be due to the lack of inhibitory effect of TIMP-3 on MMP-9. However, there are no commercial compounds that can selectively inhibit MMP-9 and ADAMs in order to assess the exact contribution of these two different proteases in colitis occurring in TIMP-3-KO mice. A decreased expression of TIMP-3 has been detected in DSS-induced colitis in rats [159] and in inflamed intestine of CD patients [149]. These data are in contrast with the up-regulation of TIMP-3 that occurs in condition of oxidative stress in macrophages and in chondrocytes [160,161]. In particular, the up-regulation of TIMP-3 expression, related to ROS production and suppressed by NAC treatment is mediated by small mother against decapentaplegic (Smad) 2 protein [161]. Considering that TGF-β1 increases TIMP-3 expression also in normal gut, it is possible that the diminished TIMP-3 expression in CD patients is in part due to Smad7, an inhibitor of TGF-β1 signalling, expressed at high levels in CD mucosa [149]. TIMP-3 decrease can be responsible of the increased paracellular permeability and tight junction protein alteration due to TNF-α [148]. Taken all, these findings suggest that TIMP-3-based therapies capable of blocking TNF-α may be considered in clinical use to control IBD in TGF-1-stimulated chondrocytes.

TIMP-4: It is shown that the levels of TIMP-4 decrease in serum of IBD patients with a consequent increase of MMP-2 activity. This may be very important, because MMP-2 is a key player in proper wound healing, angiogenesis and re-epithelization, as well as in the regulation of epithelial barrier function of the intestine [156]. TIMP-4 also inhibits platelet aggregation suggesting its involvement in the regulation of platelet recruitment and aggregation, processes enhanced in IBD patients and even in inactive disease [162,163].

Table 3 summarizes the principal roles of MMPs, ADAM17 and TIMPs in IBD

Conclusions

MMPs, ADAMs and TIMPs play an important role in wound healing and in numerous pathophysiological processes. Moreover, they are involved in the modulation of inflammatory response, by controlling migration of immune cells, matrix deposition and degradation as well as cytokine activity. However, an increase of MMPs and ADAM17, often linked to the disease severity and not accompanied by an up-regulation of TIMP, is present in intestinal mucosa of IBD patients. This can contribute to increase

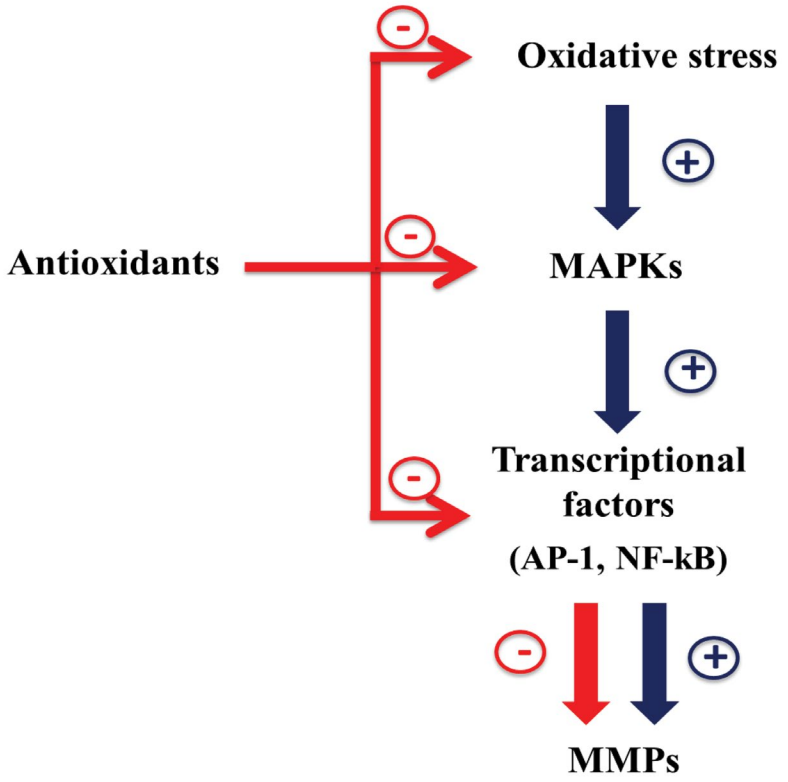

Figure 3: Down-regulation of MMPs expression by antioxidants: possible mechanisms.

the inflammatory state, cytokine production, and ulceration and fistulae formation. MMPs, differently to TIMPs, are also redox-regulated. This review highlights that the up-regulation of some MMPs production in IBD can be due to the oxidative stress present in intestinal mucosa of IBD patients. In fact, antioxidants are able to down-regulate MMPs production restoring their levels to those of control. Considering that an important role is attributed to oxidative stress in the pathogenesis of IBD, it is possible that the imbalance between oxidants and antioxidants can affect the expression of various MMPs in IBD. Moreover, antioxidants, in addition to restoring the intracellular redox state, act directly on the regulation of MAPKs and transcriptional factors (Figure 3). For this, in the development of MMP-targeting therapies, molecules with antioxidant property may be used to reduce dysfunction of epithelial barrier and prevent fistulae and ulceration in IBD.

Conflict of Interest

The authors have no conflict of interest.

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